Discovery of novel SecA inhibitors of Candidatus Liberibacter asiaticus by structure based design

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Candidatus Liberibacter asiaticus is the causal agent of Huanglongbing (HLB) disease of citrus. Current management practices have not been able to control HLB and stop the spread of HLB. The current study is focused on screening small molecule inhibitors against SecA protein of Ca. L. asiaticus. Homology modeling, structure based virtual screening and molecular docking methods have been used to find the novel inhibitory compounds against SecA activity at ATP binding region. At 20 μM compounds showed >50% inhibition and four compounds had more than 65% inhibition. The most active compound has IC50 value of 2.5 μM. The differences between the activities of the compounds are explained by their inter-molecular interactions at ATP binding site.

Huanglongbing (HLB) is the most devastating disease of citrus. HLB has seriously affected citrus production in a number of countries in Asia, Africa, the Indian subcontinent, the Arabian Peninsula, and a number of islands in the Indian Ocean, and most recently in Brazil (2004), Florida (2005), Louisiana (2008), and Mexico (2009) (USDA-APHIS). HLB disease is associated with 'Candidatus Liberibacter (Ca. L.) spp.' based on its 16S rRNA gene sequence2 and recent attempts have limited success in culturing the organism.3 There are currently three known species of Ca. Liberibacter affecting citrus: Ca. L. asiaticus, Ca. L. africanus, and Ca. L. americanus.4,5 Ca. L. asiaticus is the widest spread and most virulent strain and so far the only one reported in the US. The current management strategy of HLB is to chemically control psyllids and scout for and remove infected trees, which have not been able to control HLB and stop the spread of HLB. Thus, alternative management approaches such as treating Ca. L. asiaticus infected citrus with antimicrobial compounds are necessary. In this study, SecA protein was used as a target for structure based virtual screening and molecular docking to identify the antimicrobial compounds against Ca. L. asiaticus. SecA is an intrinsic subunit of the pre-protein translocase with ATPase activity, which is involved in pre-protein translocation across and integration into the cellular membrane in bacteria. Affecting ATP-hydrolysis process of SecA by identifying small molecule inhibitors at ATP binding site will intrude the function of SecA, thus stop the pre-protein translocation, and this could lead to the discovery of potential antimicrobial agent.

SecA is one essential component of the Sec machinery which provides a major pathway of protein translocation from the cytosol across or into the cytoplasmic membrane.6,7 Interestingly, Ca. L. asiaticus encodes one complete SecA pathway.8 SecA becomes active as an ATPase to drive protein translocation when it is bound to the SecYEG complex, acidic phospholipids and a precursor protein such as pro-OmpA (the precursor of outer membrane protein A).9,10 The crystal structures of SecA are available for other bacteria such as Escherichia coli11 and the active site/binding pocket of SecA is clearly defined. These crystal structures have been used in structure-based virtual screening and several SecA inhibitors were identified as potential antimicrobial lead compounds.6,12–14 Successful development of antimicrobial agents against Ca. L. asiaticus which are suitable for citrus application will provide one alternative approach to control HLB. On the other hand, the identified active compounds might be useful for other bacterial diseases, due to the existence of SecA gene in other bacteria.

Homology model for SecA of Ca. L. asiaticus was built with Modeller6v215 by using X-ray crystal structure of E. coli SecA homodimer bound with ATP which is reported in Protein Data Bank (PDB ID: 2FSC).11 The modeled protein structure was prepared with appropriate bond orders and formal charges by protein preparation wizard, of Maestro module in Schrodinger software.16 Then ATP ligand was manually docked as reported11 against the homology model and Ligand–Protein complexes were energy minimized with molecular minimization of Macromodel17 program.

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with AMBER force field and 'distance-dependent' dielectric constant was used during minimization. The receptor grid file was generated by excluding ATP and defining 8 Å radius from ATP without any constraints. Based on physicochemical properties, that is, Log $P$, molecular weight, H-bond donor, H-bond acceptor & rotatable bonds and structurally similar to adenine moiety, 5016 structures were retrieved from Chembridge and Specs chemical databases containing ~2.5 million structures. High throughput screening by molecular docking was performed and dock scores were calculated to filter the structures. The filtrates were subjected

Figure 1. Inhibitory effect of screened compounds against SecA of Ca. L. asiaticus. (A) Compounds 1–10 at 2 and 20 μM. (B) Compounds 11–20 at 2 and 20 μM. (C) Compounds 12, 15 and 18 are at 1, 2, 5, 20, 50, 75 and 100 μM concentrations. Thick black dotted line indicates the 50% inhibition, and the percentage (%) of the remaining ATPase activities were represented (Tables S1–S3).
to standard and extra precession docking to evaluate the scoring functions. Glide program\textsuperscript{19,20} was used to build receptor grid file and for the docking studies. All the molecular modeling studies have been performed on HP ProLiant, RedHat Linux operating system and docking postures were taken by PYMOL program.\textsuperscript{21}

Structure based design studies were performed to discover the small molecules against SecA of \textit{Ca. L. asiaticus}. The X-ray crystal structures of SecA protein in different organisms were reported in PDB. Nevertheless the crystal structure of SecA bound with ATP from \textit{E. coli} (PDB ID: 2FSG) was used to build the 3D structure of SecA of \textit{Ca. L. asiaticus}. In order to simplify of our virtual screening process, we filtered the database (Chembridge and Specs) structures based on their physicochemical properties & similarity searches and found a total of 5016 compounds. All the structures were used for virtual screening by molecular docking method.

Figure 2. Structural information of the identified active compounds 1–20 against SecA activity of \textit{Ca. L. asiaticus}. (A) Compounds 1–10 (classified as Set I). (B) Compounds 11–20 (classified as Set II).
Based on the dock scores we excluded 4516 low score structures and selected 500 for further molecular docking & minimization to evaluate the scoring functions (Dock glide scores, Hydrophilic, Hydrophobic, H-Bond). Based on scoring functions, structural diversity, and chemical intuition, 20 structures were selected for biological activity studies. The identified compounds were purchased from Chembridge (San Diego, CA USA http://www.chembridge.com/) and Specs (Wakefield, RI USA http://www.specs.net/). Overexpression and purification of SecA protein\textsuperscript{22}, screening the compounds at various concentrations\textsuperscript{23} and measurement of the remaining ATPase activity were performed as described previously.\textsuperscript{24,25} Among the 20 screened compounds, seventeen compounds including 2, 4–8, 10 and 11–20 showed >50% inhibition at 20 \(\mu\)M, four compounds 12, 14, 15 and 18 showed the highest activities with more than 65% inhibition against SecA of the \(\text{Ca}. L.\) asiaticus (Tables S1 and S2).

Based on the activity profile the 20 compounds were divided into two sets. Compounds with lower activities were defined as Set I (Figs. 1A and 2A) and compounds with higher activities were classified as Set II (Figs. 1B and 2B). The key differences between these two sets are that the Set II compounds have 40–50% inhibition at 2 \(\mu\)M and have greater than 60% inhibition at 20 \(\mu\)M (Fig. 1B). Moreover, the Set I compounds have less than 40% inhibition at 2 \(\mu\)M (Fig. 1A). To find out the inter-molecular interactions of compound 3 (which is one of the lowest in inhibition), and 18 (which is among the highest in inhibition) were docked at SecA ATP binding site with glide extra-precession and analyzed. Their binding affinities at the active site region were compared with ATP binding mode in SecA. The triphosphate group of bound ATP structure in SecA is interacting with Arg109, Thr104, Gly107, Lys108 and the adenine moiety is staking \(\pi-\pi\) interactions with Phe84 (Fig. 3A). Compound 18 is also producing similar interac-

\begin{figure}
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\includegraphics[width=\textwidth]{fig2}
\caption{(continued)}
\end{figure}
tions as ATP and the aromatic ring of the inhibitor structure was moved diagonally though it is favorable to π-π interaction with Phe84, and this is strongly supporting the high binding affinity of the compound (Fig. 3B). The low activity compound 3 has a favorable binding on the surface of the active site and loses all the interactions except one H-Bond with Gln97 (Fig. 3C). Hence it was
determined that the structures with varied binding mode and low
dock score (Table S1) at ATP binding site have lower activities
while structures with high dock scores (Table S2) have better activities.
However, significant differences were not found, between the
structural fragments of Set I & Set II compounds. The –CN groups
are only present in low activity & activity. The three high activity compounds 12, 15, and
18 from Set II were studied at various concentrations to calculate
IC50 values (Fig. 1C) and compound 18 showed IC50 value of
2.5 μM (Table S3).

In summary, virtual screening and molecular docking methods
were used to screen inhibitors of SecA of Ca. L. asiaticus, and 20 compounds
were used for activity studies. Among them, at 20 μm seventeen compounds showed >50% inhibition and four of them had more than 65% inhibition activities. One potent compound 18 was found with IC50 value of 2.5 μm. Molecular docking scores and molecular interactions were correlated with the activity of high (18) and low (3) activity compounds. The differences between the high and low activity structures, might be partially explained by the –CN groups which are only present in low activity compounds and 1, 3 di-methoxy groups which are only present in the high activity compounds. The best active structure could be utilized as a lead, to discover the novel inhibitory compounds against Ca. L. asiaticus as well as other bacterial pathogens.

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Supplementary data
Supplementary data (include the percentage of inhibitions of
ATPase activity with mean standard deviation and molecular docking
scores) associated with this article can be found, in the online version,

References and notes
21. The secA gene was first amplified by PCR using primers ACC CCC GAC CAA GGC ATC CA and AGG GGA AGA TGA GGC GTT AGG with genomic DNA of Ca. L. asiaticus str. psy62 as template. The amplification product was gel purified and re-amplified by using primersCAC CCC GAC CAA GGC ATC CA and ACC CCC GAC CAA GGC AGT GC with genomic DNA of Ca. L. asiaticus str. psy62 as template. The amplification product was gel purified and sub-cloned into the pE-SUMO vector (LifeSensors, Malvern, PA) downstream of the poly-histidine coding region. The SecA protein was overexpressed in E.coli BL21DE3 host cells (Stratagene, La Jolla, California). Cultures were induced with 0.2 mM IPTG and incubated at 37°C for 8 hours. The protein was purified as following: harvested cells were resuspended in a lysis buffer of 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride, 20 mM Imidazole and 1 Tabl/titer Complete Protease Inhibitor Cocktail (EDTA FREE). The cells were lysed by Sonicator on ice bath. The lysate was loaded onto a 5.0 ml Ni-Column. The bound protein was eluted with 25–200 mM imidazole. The peak fractions containing the target protein were concentrated. The protein was loaded onto a Superdex 200 column (GE Health Care, Waukesha, WI) equilibrated with 10% glycerol, 150 mM NaCl, 2 mM DTTS, 25 mM Tris-HCl (pH 8.0). The fractions contained 95% purity of SecA were collected and concentrated to 0.25 mg/ml and stored at −80°C.
22. Quantichrom ATPase/GTase kit (BioAssay Systems, Hayward, CA) was used to test the inhibitory effect of different compounds on SecA of Ca. L. asiaticus. Briefly, the small molecule compounds were dissolved in DMSO to make a 100 μm stock solution and kept at room temperature and then diluted to required concentrations. The total reaction mixture is 50 μL contains 20 μl assay buffer, 10 μL Enzyme, 10μl 2mM ATP, 10μl of inhibitor. The micro plate is incubated at 40°C for 40 min and 200 μL of Malachite Green (MG) and then 25 μL of 34% (w/v) of sodium citrate was added to stop unwanted hydrolysis of ATP caused by Mg2+ content of MG solution. Then the plate was incubated at room temperature for 40 min and then the absorptions were measured at 660 nm wavelength. All these assays were done at least in triplicate. The OD values were measured from Benchmark plus ELISA microplate spectrophotometer (Bio-RadHercules, CA). ATPase activities were determined by the release of phosphate ion (Pi) detected spectrophotometrically using malachite green and inhibition is calculated by showing the percentage (%) of the remaining ATPase activities.