Characterisation of *Staphylococcus aureus* virulence factor EsxA and structure-based screening of EsxA inhibitors for combating methicillin-resistant *S aureus*: abridged secondary publication

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KEY MESSAGES

1. EsxB did not interact with EsxA by cell-free assays (ITC and NMR titration) and cell-based assays (coIP and pull-down).
2. Pull-down and NMR titration assays showed that EsxA was interacting with lipid mediators HOTrE and sphingosylphosphorylcholine, respectively. These results suggested that EsxA may function as a lipid mediator binding protein, and that EsxA is an immune evasion gene and provide important clue to delineate its mechanism of immune evasion.
3. A high performance platform was established for *in silico* structure-based screening against pathogen targets. The EsxA X-ray structure was subjected to structure-based screening with a ligand library containing 6.8 million lead-like or active lead ligands.
4. Of the 100 highest-scoring compounds, five were validated by the secondary NMR titration screen. One hit compound (6058448) showed MIC at 25 μM level by broth microdilution test, and another hit compound (5674203) also showed antivirulence effects by inhibiting the expression of both protein A and alpha-toxin of US300 strain.

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Introduction

Staphylococcus aureus is a highly adaptive Gram-positive bacterium and commensal of the human skin and nostrils. *S aureus* is a common cause of minor skin and wound infections, but it can also cause serious and even fatal infections, particularly in immunocompromised persons. Methicillin-resistant *S aureus* (MRSA) infection is an alarming threat, leading to life-threatening diseases such as endocarditis, pneumonia, and toxic shock syndrome.[1] MRSA has been endemic in Hong Kong since mid-1980s. Approximately 70% and 58% of the total and blood culture isolates of *S aureus* in Hong Kong public hospitals are MRSA, respectively. There is a need to develop new approaches and druggable targets for combating MRSA.

S aureus pathogenesis depends on a specialised protein secretion system (type VII-like Ess) that delivers a range of virulence factors to assist infectivity by establishment of abscess lesions and suppression of host immune responses. EsxA and EsxB are two of the confirmed virulence factors excreted by *S aureus*.[2] EsxA is reported to be an immune evasion gene but how it can achieve this function remains unclear.[3] EsxA and EsxB are small α-helical polypeptides belonging to a family of WXG100 motif proteins, which have a size of approximately 100 amino acids containing a helical structure and a conserved Trp-Xaa-Gly (WXG) motif. ESAT-6 (homologue of *S aureus* EsxA) and CFP-10 (homologue of *S aureus* EsxB), secreted as a heterodimer by *Mycobacterium tuberculosis*, are the founding members of the WXG100 motif family. Both proteins are crucial for the replication of *M tuberculosis* in macrophages and presumably also for the pathogen's ability to suppress innate and adaptive immune responses. Mutants that failed to secrete EsxA and EsxB displayed defects in the pathogenesis of *S aureus* murine abscesses, suggesting that these specialised secreted WXG100 motif proteins may be a general strategy of human bacterial pathogenesis.[2] Therefore, EsxA and EsxB are promising drug targets for combating MRSA and may have add-on implications in fighting against other drug-resistant pathogens such as multidrug-resistant strains of *M tuberculosis*.

A homodimeric structure of EsxA has a four-helix bundle fold.[4] The structures of EsxB and the

putative EsxA-EsxB complex remain unknown. The actual role of EsxA and molecular basis for its function remain unclear. The expectation that EsxA will be secreted by forming a heterodimer with EsxB in analogy with the well-characterised ESAT-6/CFP-10 heterodimeric complex in *M tuberculosis* is also illusive. Therefore, characterisation of the binding of *S aureus* EsxA with EsxB and identification of other EsxA interacting partners or small substrates will provide important information in understanding the functional role of EsxA.

This pilot study aims to characterise the interaction of *S aureus* EsxA with EsxB by cell-based and cell-free methods and to perform in silico structure-based screening to identify inhibitors of EsxA for combating MRSA.

Methods and Results

Virulence factors EsxA and EsxB of *S aureus* were cloned, and their labelled and unlabelled His-tagged recombinant protein samples were produced for cell-free and cell-based interaction studies.

The backbone resonance assignments of EsxA were completed using a series of 3D triple resonance NMR experiments to allow performing interaction studies of labelled EsxA with small ligand hit compounds, lipid molecules and EsxB using NMR chemical shift perturbation titration method.

Cell-free titration assays including ITC and NMR indicated that no *in vitro* interaction between the recombinant EsxA and EsxB samples (Fig 1). Cell-based coIP of EsxA against US300 infected J774 macrophages and pull-down assays of EsxA indicated that EsxB was not directly binding with EsxA. Instead, a lipid mediator molecule HOTrE was found in the pull-down extract of EsxA, indicating EsxA may be a lipid-binding protein. Subsequent *in vitro* NMR titration assay indicated that another lipid mediator, sphingosylphosphorylcholine, could also bind to EsxA. These results support that EsxA is a lipid-binding protein.

Two dedicated Dell OptiPlex 9020 workstation computers with Intel Core i7-4770 Quad Core Processor and 32GB Ram were setup with Fedora 15 Linux operating system and all required software for running Autodock/Vina program. These workstations have established a dedicate research platform and the capacity for performing *in silico* structure-based screening on a target protein. A compound library containing 6 746 897 Lead-Like compounds from ZINC database and 50 240 compounds from active lead library of ChemBridge Corp was setup for the screening.

Autodock/Vina dockings were completed on all 6.8 million ligands in the library against the X-ray structure 2VS0 of EsxA. Scoring sorting and clustering analysis by AuPosSOM of the best-hit ligands according to their structural features were performed in order to draw up the list of best docking scoring ligands for *in vivo* validation and testing. Fig 2 shows the AuPosSOM scoring plots and the graphical representation of the major predicted contacts.

Of the 100 highest-scoring compounds, five produced significant cross-peak movements or intensity variations upon titration into ^{15}N labelled EsxA in secondary experiment screen of NMR titration assay.

The broth microdilution test showed that hit compound 6058448 showed MIC at 25 μM, whereas hit compounds 5224045, 5546503, and 6238413 have MIC >100 μM.

Hit compound 5674203 showed significant antivirulence effects by inhibiting the expression of both protein A and alpha-toxin, whereas hit compounds 5224045, 5546503, 6058448, and 6238413 did not show any inhibitory effects (data not shown).

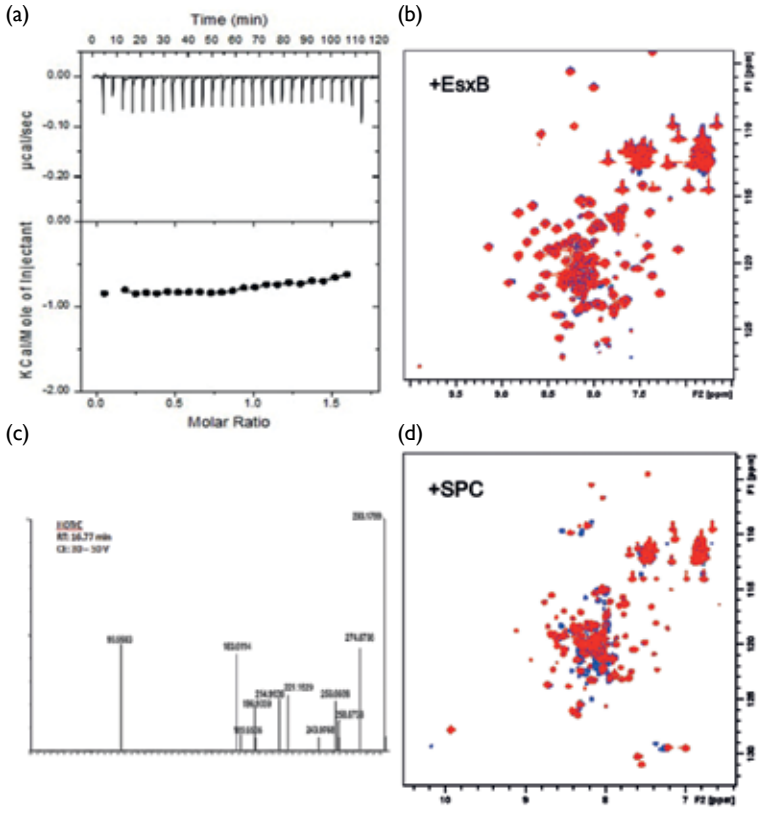


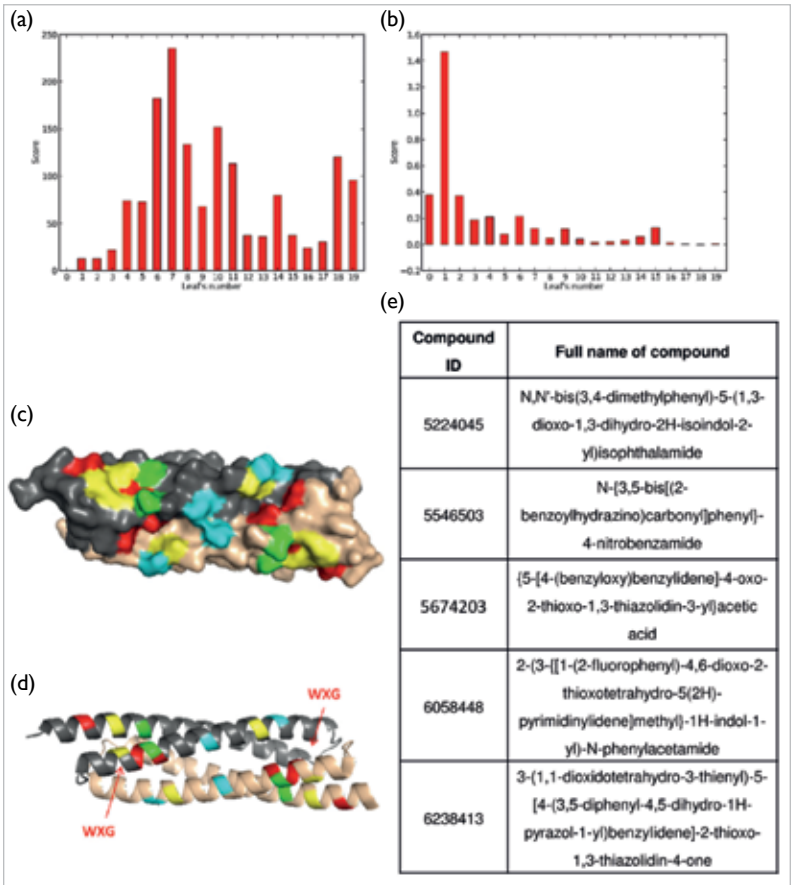
FIG 1. Interaction assays on EsxA: (a) thermogram for the ITC titration of 1 mM His-tagged EsxA with a 100 μM solution of His-tagged EsxB. (b) ^{1}H-^{15}N HSQC NMR spectra of titrating unlabelled EsxB against 0.3 mM ^{15}N labelled EsxA (red: 2D spectrum of apo-EsxA sample; blue: 2D spectrum with addition of 2 molar equivalent of EsxB sample). (c) MS/MS characterisation of identified lipid molecule HOTrE by LC-MS analysis from the pull-down substrate extract of EsxA. (d) ^{1}H-^{15}N HSQC spectra of titrating sphingosylphosphorylcholine (SPC) against ^{15}N labelled EsxA (red: 2D spectrum of apo-EsxA sample; blue: 2D spectrum with addition of one molar equivalent of SPC).

ITC experiment has been performed using recombinant EsxA and the validated hit compound 5674203 with antivirulence activity. The thermogram was best fitted with one-site model to generate a binding affinity Kd value of 90.0 μM, N=0.5, ΔH= -59.6 kcal/mol, ΔS= -186 cal/mol/deg.

Discussion

EsxB did not interact with EsxA. Instead, EsxA was shown to be a lipid mediator binding protein. EsxA is a secreted virulence factor by *S aureus* implicated to be an immune evasion gene, but its mechanism of immune evasion remains unknown.[3] The ability of EsxA to trap lipid mediators may provide clues about its immune evasion function. We have reported that a highly secreted and helical virulence factor Mp1p of fungal pathogen, *Talaromyces marneffei*, binds and sequesters a key proinflammatory lipid (arachidonic acid) to dampen host innate immune response.[5] Therefore, bacterial pathogens may have a similar host immune evasion mechanism. The function for the highly secreted and helical WXG100 motif virulence factor proteins found in many bacterial species remains unclear. It is of interest to investigate whether other WXG100 motif virulence factors also serve as lipid-binding proteins to evade host immune defence. Our preliminary NMR titration data obtained on the heterodimeric WXG100 motif ESAT-6/CFP-10 of TB showed that it is also able to bind sphingosylphosphorylcholine.

We have established a dedicated Linux computer platform for *in silico* structure-based screening for drug targets with known high-resolution structures. MRSA is of great concern because it is quickly acquiring resistance to all clinical antibacterial agents. There is an urgent need to develop new approaches and new druggable targets to combat MRSA with lower chances of developing drug resistance. In this study, we identified several hit compounds against MRSA through *in silico* structured-based screening against the novel target EsxA of *S aureus*. These hit compounds likely act against MRSA through a mechanism different from the existing antibiotics, because EsxA is a virulence factor of *S aureus*. For example, hit compound 5674203 showed no antibacterial effect but significant antivirulence effects to enhance host cell protection. Antivirulence drug against *S aureus* infection with diminished virulence will cause less or no damage to the host cells and tissues and will not subject to natural selection pressure so that the antivirulence drug is less likely to cause drug resistance. Through the hit-to-lead process, these hit compounds can be further modified and improved to attain higher potency and better pharmacokinetics properties. The improved compounds could be the candidates for new drug development against MRSA.



Compound ID	Full name of compound
5224045	N,N'-bis(3,4-dimethylphenyl)-5-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)isophthalamide
5546503	N-[3,5-bis[(2-benzoylhydrazino)carbonyl]phenyl]-4-nitrobenzamide
5674203	{5-[4-(benzyloxy)benzylidene]-4-oxo-2-thioxo-1,3-thiazolidin-3-yl}acetic acid
6058448	2-(3-{[1-(2-fluorophenyl)-4,6-dioxo-2-thioxotetrahydro-5(2H)-pyrimidinylidene]methyl}-1H-indol-1-yl)-N-phenylacetamide
6238413	3-(1,1-dioxidotetrahydro-3-thienyl)-5-[4-(3,5-diphenyl-4,5-dihydro-1H-pyrazol-1-yl)benzylidene]-2-thioxo-1,3-thiazolidin-4-one

FIG 2. Clustering analysis of docking results and list of validated hit compounds: (a) AuPosSOM scoring plot using all contacts for analysis. Each leaf number corresponds to a particular cluster of compounds with similar binding modes. A high positive score indicates high chance of finding active compounds inside the leaf. (b) AuPosSOM scoring plot using hydrogen bonding contacts for analysis. (c) Graphical representation of major predicted contacts in surface plot of EsxA dimer structure (PDB: 2VS0). (d) Graphical representation of major predicted contacts in surface plot (red: major contacts exclusive to leaf 6 of all contacts main tree; green: major contacts exclusive to leaf 7 of all contacts main tre; yellow: major contacts discovered in both leaf 6 and 7 of all contacts main tree; cyan: major contacts that are exclusively found in hydrogen bond analysis). (e) List of hit compounds that displayed binding effect in secondary experiment screen by NMR titration assay with EsxA.

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