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Flavonoid Eriodictyol Struts as an Anti-virulent Sortase A Inhibitor.

Katherine Liew-Tan
La Trobe University, La Trobe Institute For Molecular Science

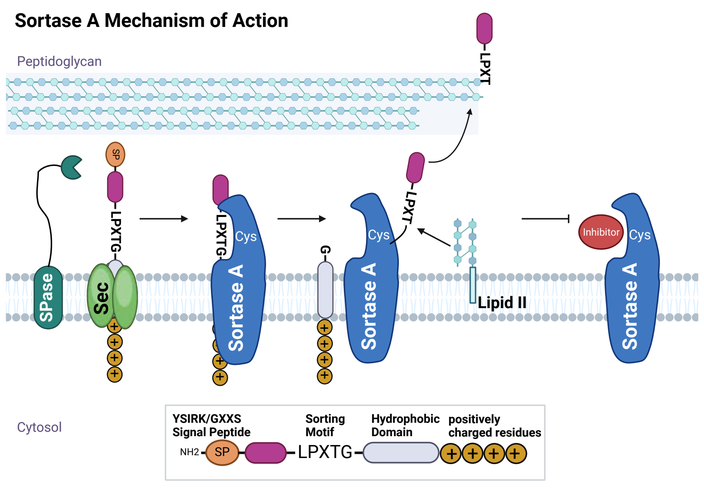
INTRODUCTION

Anti-virulence approach to rising antibiotic resistance

Methicillin-resistant *S. aureus* (MRSA) is identified as a high-priority threat by WHO, causing more than 100,000 death globally in 2019[1]. *S. aureus* is a leading cause for pneumonia, bacteremia and endocarditis [2]. Anti-virulence approach is an alternative to combat antibiotic-resistant pathogens by disarming pathogens' virulence factors without killing them in order to attenuate the pathology of diseases. This approach imposes a lower selection pressure for resistance development.

Staphylococcus aureus Sortase A as an anti-virulence target.

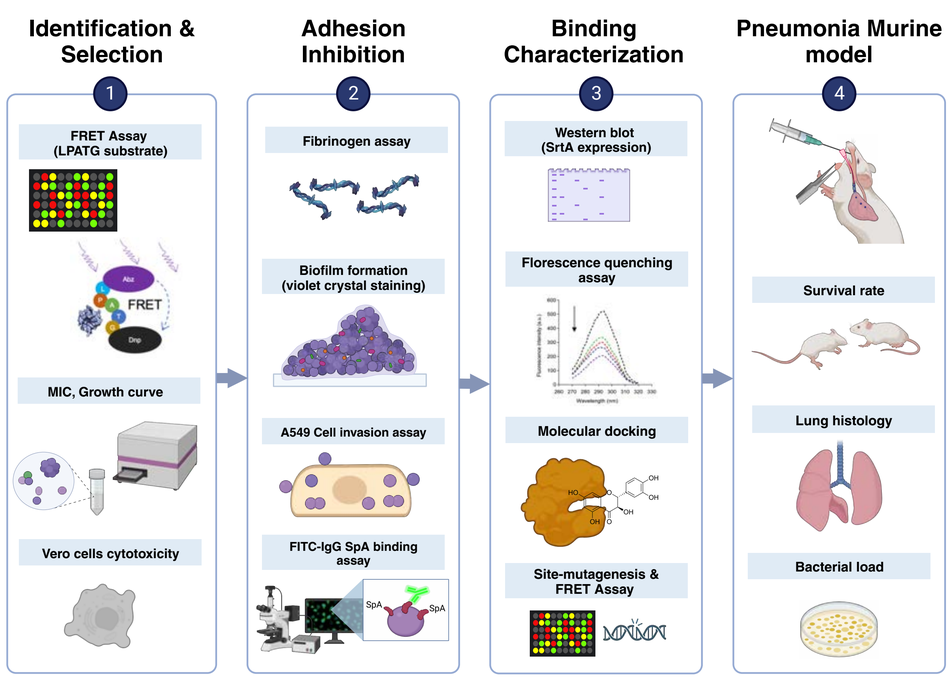
S. aureus is armed with an arsenal of virulence factors on its surface to advance its pathogenesis, initiating with the critical step of adhesion. These include important surface-associated adhesins such as fibronectin-binding proteins (FnBPs) and staphylococcal protein A (SpA).[2] Importantly, what hinges these LPXTG-motif virulence-related proteins on the peptidoglycan cell wall is Sortase A (SrtA), a membrane cysteine transpeptidase that necessitates their anchoring.[2] SrtA cleaves between threonine (T) and glycine (G) to form an acyl enzyme intermediate. It is further catalysed to form an amide bond with the cross-bridge within lipid II (a membrane-bound peptidoglycan precursor) and then joins the forming peptidoglycan.



Aim

Flavonoid eriodictyol was identified as a potential SrtA inhibitor from the natural compound library. This research aimed to investigate its inhibitory ability, binding mechanism as well as to evaluate its therapeutic effect on an MRSA-induced pneumonia-mouse model.

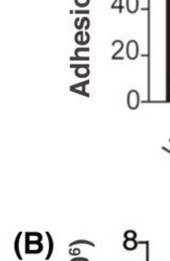
METHODS



The methodology approach consists of four key pillars, covering both *in vitro* and *in vivo* experiments

1. A high-activity SrtA inhibitor (eriodictyol) was identified from natural compound library screening using fluorescence resonant energy transfer (FRET). Qualification of eriodictyol as an anti-virulent agent was assessed by its minimum inhibition concentration, non-toxicity, binding reversibility and impact on *S .aureus* growth.
2. *In vitro* adhesion inhibiting capability of eriodictyol was investigated through fibrinogen binding assay, biofilm formation test, cell invasion assay and FITC-IgG binding assay for SpA.
3. Characterization of the binding between SrtA and eriodictyol were investigated with western blot, fluorescence quenching assay, molecular docking analysis and subsequent site-mutants with FRET assay.
4. *In vivo* pneumonia-mouse model infected with methicillin-resistant *S. aureus* (USA300) was used to investigate eriodictyol's therapeutic effect.

RESULTS



Identification and Qualification

- Eriodictyol is a non-toxic reversible inhibitor of SrtA with IC_{50} of 2.229 µg/mL ± 0.014 mg/mL and MIC > 512 µg/mL. It did not inhibit *S. aureus* growth even at 128 µg/mL.

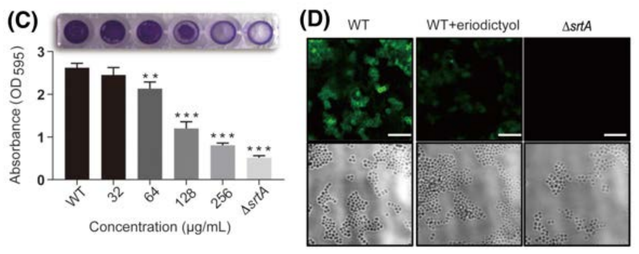
Binding Characterization

- Eriodictyol binds directly to SrtA with good affinity, with constant K_A at 8.2×10^4 L/mol.
- The four binding sites were identified as Leu-97, Aka-104, Ile-182 and in particular Arg-197. His-120, Cys-184 and Arg-197 are catalytic sites of Sortase A.[4]

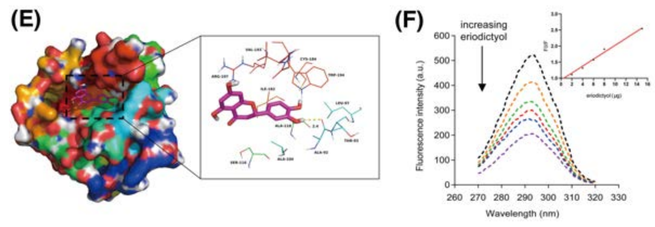
Adhesion Inhibition Activity

- Eriodictyol reduced >60% adhesion rate ($P<0.001$) of the untreated WT at 128 µg/mL.
- At same concentration, it suppressed the cell internalisation of *S. aureus* by one-third ($P<0.01$).
- Correspondingly, biofilm biomass was reduced by half at 128 µg/mL ($P<0.001$)
- SpA anchoring on the bacteria cell surface was visibly decreased at 256 µg/mL.

(A) Eriodictyol inhibited adhesion of *S. aureus* USA300 to fibrinogen. (B) Eriodictyol suppressed the internalization of *S. aureus* USA 300 into A549 cells. The data are shown as the mean SEM (error bars) of three replicates. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the WT (wild-type) un-treated group according to the two-tailed Student's t-test. (ΔsrtA) is a SrtA deletion mutant and negative control.



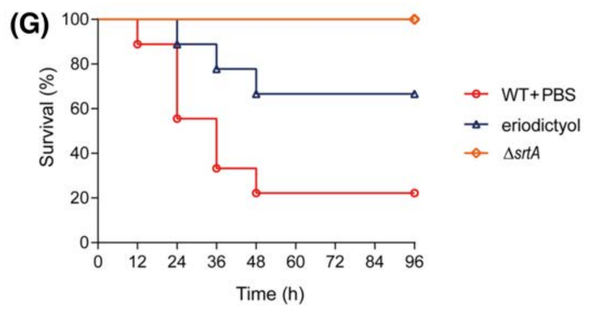
(C) Eriodictyol inhibited biofilm formation of *S. aureus* USA300, quantified by the crystal violet (CV) staining assay. (B) Confocal laser microscopy analysis of surface-anchored SpA proteins stained with FITC-labeled rabbit IgG. Magnification: 600; scale bar, 50 mm. The data are shown as the mean SEM (error bars) of three replicates. ** P < 0.01, *** P < 0.001 vs. the WT untreated group according to the two-tailed Student's t-test. (ΔsrtA) is a SrtA deletion mutant and negative control.

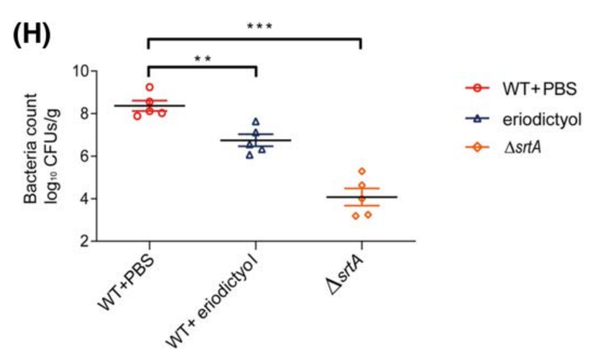


(E) Molecular modelling of the interaction of eriodictyol with SrtA. (F) Fluorescence quenching assays were performed to evaluate the binding affinity of eriodictyol to SrtA.

In-vivo Pneumonia-mouse Model

- Eriodictyol-treated mice showed a significantly higher survival rate (> 60%) than untreated mice (20%). Furthermore, there was visibly less histopathology and significantly less ($P < 0.01$) bacteria load in the lung.





For fig (G-I), 7-week-old female mice were intranasally lethally-infected with *S. aureus* USA300 and treated with 100 mg/kg of eriodictyol on a 12-hour cycle for 8 days. Comparison with WT PBS-treated. (ΔsrtA) is a SrtA deletion mutant and negative control. (G) Survival rate of mice (n = 10). **P < 0.01; log-rank test. (H) Bacterial load in the lungs of mice (n = 5). **P < 0.01, ***P < 0.001; Mann-Whitney test, two-tailed. Horizontal bars represent the mean values. (I) Histopathology of the lung (H&E staining) after 24 hrs. (magnification: 400; scale bar, 50 mm). Animal data in (I) was obtained in separate experiment.

CONCLUSIONS

- Flavonoid eriodictyol has demonstrated its attenuating ability of *S .aureus* pathogenesis, particularly with one key binding site at SrtA catalytic centre and low IC_{50}. It is a promising as an anti-virulence candidate for future development. Our next step is to investigate eriodictyol's pharmacokinetics and verify the molecular binding with X-ray crystallography and SPR.
- Eriodictyol also has potential for wider coverage as Sortase A is highly conserved across gram-positive bacteria.[5]
- We proposed a standardization of methodological approach and results reporting which hopefully facilitates cross-study comparison and further build upon the research knowledge of anti-SrtA anti-virulent agents.

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Special acknowledgement to Dr Pramod Subedi, Dr Fung Lay, Dr Seah Seng Wee and Dr Falicia Goh.