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Molecular basis of *Streptococcus mutans* sortase A inhibition by the flavonoid natural product *trans*-chalcone†

Daynea J. Wallock-Richards,† Jon Marles-Wright,‡ David J. Clarke,§ Amarnath Maitra,¶ Michael Dodds,‖ Bryan Hanley and Dominic J. Campopiano*†

Sortase A (SrtA) from Gram positive pathogens is an attractive target for inhibitors due to its role in the attachment of surface proteins to the cell wall. We found that the plant natural product *trans*-chalcone inhibits *Streptococcus mutans* SrtA in vitro and also inhibited *S. mutans* biofilm formation. Mass spectrometry revealed that the *trans*-chalcone forms a Michael addition adduct with the active site cysteine. The X-ray crystal structure of the SrtA H139A mutant provided new insights into substrate recognition by the sortase family. Our study suggests that chalcone flavonoids have potential as sortase-specific oral biofilm inhibitors.

Sortases are a family of membrane-associated transpeptidases which are highly conserved in Gram positive bacteria. They are a promising target for inhibitors which have the potential to be used as anti-infective therapies against pathogenic bacteria.1–3 Various sortase isoforms (SrtA, B, C) are responsible for covalently attaching surface proteins involved in adherence to host cells, iron acquisition, biofilm formation, invasion, signalling and pilus formation on the bacterial cell wall.2,4,5 Sortase A (SaSrtA) was the first isoform of this family identified in *Staphylococcus aureus* by Schneewind and colleagues, who also elucidated the catalytic mechanism of the enzyme.6–10 The SrtA enzymes recognize a conserved five amino acid sequence (LPXTG) at the C-terminus of their protein substrates and cleave the amide bond between the threonine and glycine residues using a highly-conserved catalytic triad (Cys–His–Arg) which stabilises the oxoanion intermediate formed during the reaction.11–13 This catalytic triad (Cys–His–Arg) is highly conserved among the sortase A enzymes (Fig. S2, ESI†). Several classes of SrtA inhibitors have been identified and include compounds from small-molecule synthetic libraries,9,11,14,15 rationally designed peptidomimetics14,16 and natural products.17–20 Flavonoids21 are polyphenolic plant natural products and two in particular, morin22 and curcumin,23,24 display good inhibition against *Streptococcus mutans* SrtA. However, the exact molecular basis of their activity has not been determined. Combining the knowledge gained from these previous inhibitor studies we hypothesized that *trans*-chalcone (Fig. 1A, inset), the precursor molecule of many flavonoids, would inhibit the *S. mutans* SrtA in vitro and possibly prevent bacterial biofilm growth.

The *S. mutans* SrtA enzyme was expressed in *E. coli* as an N-terminal 40 amino acid truncation to remove the predicted transmembrane domain (Fig. S3 and S4, ESI†) and the isolated SrtA had a mass of 22 767 Da ([M + H]+, Fig. S5, ESI†) which agrees well with the predicted value based on the sequence.25 The established FRET-based assay was used to measure the *K_m* and *V_max* values (90.4 ± 4.7 μM and 4.46 ± 0.11 × 10⁻⁴ μM s⁻¹ respectively) for cleavage of the fluorogenic peptide substrate, dabcyl-QALPETGEE-edans (Fig. S6, ESI†).26 Using the published method,24 an IC₅₀ value of 5 ± 0.6 μM was determined for *trans*-chalcone after incubating SrtA with varying concentrations of inhibitor overnight (Fig. 1A and Fig. S7, ESI†). The rate of inhibition was measured in a time dependent study by incubating 5 μM of the enzyme with 100 μM of the inhibitor for various times (1, 2, 4, 8 and 16 h, Fig. 1B). Analysis of the enzyme activity revealed that the inhibition of SrtA was slow, taking up to 16 h to reduce activity by 90%. Enzyme activity was not restored upon extensive dialysis of the enzyme (not shown). Taken together these observations suggested that *trans*-chalcone was a slow and tight binding inhibitor of SrtA.

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‡ These authors contributed equally.

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a EastChem School of Chemistry, The University of Edinburgh, David Brewster Road, Edinburgh, EH9 3JF, UK. E-mail: Dominic.Campopiano@ed.ac.uk
b Institute of Structural and Molecular Biology, The University of Edinburgh, Edinburgh, EH9 3BF, UK.

† Wm. Wrigley Jr. Company, 1132 W. Blackhawk Street, Chicago, IL 60642, USA

‡ The Knowledge Transfer Network, The Roslin Institute, Easter Bush, Roslin, EH25 9RG, UK.
This led us to postulate that a covalent adduct was formed between the inhibitor and the SrtA. To test this hypothesis, mass spectrometry was used to analyse both the concentration and time dependence of the reaction. From the mass spectrum we observed a peak of 22 767 Da for the mass of SrtA and an additional peak at mass 208 Da. (D) Modification of 5-trans-chalcone with trans-chalcone (0, 20, 40, 80 and 100 μM) was concentration dependent.

Fig. 1 (A) Inhibition of SrtA by trans-chalcone (inset) after incubation with varying concentrations of the inhibitor. The IC50 of trans-chalcone is 5 ± 0.6 μM. (B) Inhibition of SrtA by 100 μM trans-chalcone over time (1, 2, 4, 8 and 16 h) (C) incubation of 5 μM SrtA (22 767 Da) with 100 μM trans-chalcone leads to a time-dependent formation of a SrtA–chalcone adduct (22 975 Da, Δmass 208 Da). (D) Modification of 5 μM SrtA with trans-chalcone (0, 20, 40, 80 and 100 μM) was concentration dependent.

As the relative positions of the active site residues of SrtA (Cys205, Arg213) are highly conserved across the family, with no architectural disparities, we attempted to gain structural insight into the SrtA:inhibitor interaction but we noticed that the wild-type SrtA enzyme was unstable during crystal trials due to auto-proteolysis (Fig. S12A, ESI†). To overcome this we produced and crystallised the more stable SrtA H139A mutant (Fig. S12B, ESI†) and, as expected, although this mutant was inactive in the in vitro fluorescent assay, we still observed the formation of the trans-chalcone adduct upon incubation of the SrtA H139A mutant enzyme with the inhibitor (Fig. S13, ESI†). This suggests that the mutant undergoes a similar Michael addition to the wild-type SrtA. Crystals of the SrtA H139A mutant defracted to 1.6 Å (Fig. S14, ESI†) and the crystal structure (PDB code: 4TQX) was determined by molecular replacement using the structure of the S. pyogenes SrtA as the starting model (PDB code: 3FN5). The electron density map at residues 49–53 at the N-terminus was ambiguous and suggests some degree of flexibility in this region; therefore these residues were omitted from the final model. The S. mutans SrtA has a canonical eight stranded β-barrel core (Fig. 2A, Fig. S15A, ESI†) which is highly conserved among the srtase superfamily.

Our construct lacks the predicted N-terminal forty amino acids of the trans-membrane domain but the structure revealed a unique, extended N-terminal helix (residues 69–89, relative to the full-length enzyme) which is absent from all other srtase A structures present in the PDB (Fig. S15B, ESI†). This helix is extended away from the catalytic domain but connected to it by a short loop (residues 89–93). An N-terminal helix was observed in the crystal structure of S. aureus SrtB (PDB: 1NG5). However, in contrast to our structure, this helix is hinged at Asp41 in SrtB and places it in an equivalent position to the C-terminus of SrtA (Fig. S15A, ESI†). This is the first observation of the SrtA N-terminal domain and its structure suggests that it plays an important functional role in positioning the catalytic domain of srtases above the membrane where it can efficiently interact with its substrates.

The relative positions of the active site residues of SrtA (Cys205, Arg213) are highly conserved across the family, with no architectural compromises evident by introduction of the H139A mutation (Fig. S15C, ESI†). The only significant difference is observed between the cysteine residue of S. aureus SrtB (SaSrtB) and the other srtase enzymes. In SaSrtB, the cysteine residue points towards the histidine residue in the active site, whereas in the other srtases the cysteine residue points away from the His separated by a distance of ~7 Å. In the crystal structure of the SrtA H139A mutant, Cys205, Ala139 and Arg213 are positioned on three neighboring β-strands, β-7, β-4 and β-3 respectively which form a tunnel-like hydrophobic pocket, similar to that described in other SrtA enzymes which are thought to adopt a conformation that is ideal for receiving the natural peptide substrate. Interestingly, when viewing the unit cell, the N-terminal α-helix (residues 69–89) from a symmetry-related molecule, makes extensive

disruptor, was used as a positive control. We observed that biofilm formation was reduced in the presence of trans-chalcone in a concentration dependent manner up to 250 μM, with efficacy tailing off at higher concentrations (Fig. S11, ESI†). Since biofilm formation is a complex and dynamic process that involves changes in microbial metabolism and signaling, this encouraging result suggests that the exact mechanism of chalcone inhibition in vivo requires further in-depth study.

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contacts with the active site cleft/binding groove of SrtA (Fig. 2A). These serendipitous “non-physiological dimers” involving the active site offer a structural rationale as to why the wild type SrtA undergoes auto-proteolysis. Although it does not contain a canonical “sortase motif” it appears that the N-terminus of SrtA binds to the active site and this self-recognition leads to amide bond cleavage.

Although we prepared milligram quantities of chalcone-modified SrtA H139A it was difficult to handle at high concentrations and failed to crystallise. Recently, Schneewind and colleagues were able to identify a new class of mechanism-based inhibitors, aryl(-)-chalcone bound to Cys205 of SrtA in both S. mutans and B. anthracis which irreversibly modify the active site cysteine residue of SrtA and provide a possible strategy for exploiting more structurally diverse chalcones drawn from the flavonoid family as isoform-specific sortase inhibitors.

In summary, our study provides strong evidence that trans-chalcone inhibits S. mutans SrtA by covalently modifying its target and also reveals novel aspects of the structure and mechanism of the sortase family. Biofilm inhibition data also lends support for further efforts into potential uses of natural products to control the growth of oral flora.

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Notes and references