An engineered protein-based submicromolar competitive inhibitor of the *Staphylococcus aureus* virulence factor aureolysin

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Abstract

Aureolysin, a secreted metallopeptidase (MP) from the thermolysin family, functions as a major virulence factor in *Staphylococcus aureus*. No specific aureolysin inhibitors have yet been described, making this an important target for the development of novel antimicrobial drugs in times of rampant antibiotic resistance. Although small-molecule inhibitors are currently more common in the clinic, therapeutic proteins and peptides (TPs) are favourable due to their high selectivity, which reduces off-target toxicity and allows dosage tuning. The greater wax moth *Galleria mellonella* produces a unique defensive protein known as the insect metallopeptidase inhibitor (IMPI), which selectively inhibits some thermolysins from pathogenic bacteria. We determined the ability of IMPI to inhibit aureolysin in vitro and used crystal structures to ascertain its mechanism of action. This revealed that IMPI uses the “standard mechanism”, which has been poorly characterised for MPs in general. Accordingly, we designed a cohort of 12 single and multiple point mutants, the best of which (I57F) inhibited aureolysin with an estimated inhibition constant (K_i) of 346 nM. Given that animals lack thermolysins, our strategy may facilitate the development of safe TPs against staphylococcal infections, including strains resistant to conventional antibiotics.

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1. Introduction

Antibiotic resistance is a major global health burden, leading to hundreds of thousands of deaths every year and greatly increasing healthcare costs associated with the treatment of bacterial infections [1–3]. Resistance arises from selection pressure caused by the widespread abuse, overuse and misuse of antibiotics in humans, including premature treatment discontinuation [4], subtherapeutic dosing, and the distribution of counterfeit drugs [5]. Furthermore, ~80% of all antimicrobials used in the USA are administered as prophylactics to farm animals to boost their health and productivity [6]. Once acquired, resistance is spread by horizontal gene transfer, often across species barriers, ultimately giving rise to multidrug-resistant strains [7]. The impact of antibiotic resistance is heightened by the lack of new drugs in the development pipeline, with only two new classes of antibiotics approved in the last 30 years: the oxazolidinones, which target protein synthesis, and the acidic lipopeptides, which target bacterial membranes [8,9]. This lack of progress reflects decades of low returns compared with other drug classes, discouraging investment by the pharmaceutical industry [2,7,10] and thus posing a serious

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threat to public health [11]. There are few therapeutic options for the treatment of infections with “superbugs” such as Acinetobacter baumannii, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Streptococcus pneumoniae and Staphylococcus aureus, which kill someone every 15 min in the USA [12–14]. Drug-resistant strains of S. aureus cause severe endocarditis, pneumonia, sepsis, and toxic shock syndrome [15]. Thus, there is an urgent need for the development of new classes of antibiotics to tackle such infections.

Microbial pathogenesis involves diverse pathways and mechanisms that lead to host colonisation and infection [16]. Virulence factors are secreted by the pathogen to facilitate this process, including peptidases that break down host defence proteins, regulate the availability of other secreted bacterial factors, and provide peptide nutrients for the pathogen. One example is the theromolysin family of bacterial metallopeptidases (MPs), also referred to as the M4 family according to the MEROPS database (www.ebi.ac.uk/merops) [17]. The archetype is Bacillus thermoproteolyticus theromolysin, which was the first endo-MP to be structurally resolved [18] and the founding member of the gluucin clan of MPs [19,20]. Related MPs produced by human pathogens include P. aeruginosa pseudolysin [21], vibriolysin from several Vibrio species [22], Burkholderia cenocepacia ZmpA/B [23], Enterococcus faecalis cocolysin [24], Legionella pneumophila Msp [25], Clostridium perfringens l-toxin [26], and aureolysin from Staphylococcus epidermidis and S. aureus [16,27–29].

Aureolysin was discovered in S. aureus strain V8 [30] and is the product of the aur gene, which is located on a monocistronic operon [31] and regulated by the alternative sigma factor σE and the staphylococcal accessory regulator SarA [31]. Aureolysin is prevalent in both pathogenic and commensal S. aureus strains [32], and peak abundance occurs during post-exponential growth and when the bacterial cells are phagocytosed by human neutrophils [33]. The enzyme accounts for ~50% of the total peptidase activity in culture supernatants [28] and participates in the extracellular peptidase system of S. aureus by activating the V8-type serine peptidase SspA, which in turn activates the cysteine peptidase SspB [16]. Together with the cysteine peptidase SspA, they constitute the four major extracellular peptidases of S. aureus [34] known as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. Moreover, aureolysin recruits nutrients from host proteins [35] and contributes as the “staphylococcal proteolytic cascade” [31]. 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The inhibitors were dialysed overnight with His 6-tagged TEV peptidase, 3 mM reduced glutathione, pH 8.0) and centrifugation (50,000 g; 1 h; 4 °C). Protein-containing fractions were dialysed and eluted on a separate column to avoid cross-contamination. IMPI HP column (Cytiva), previously washed and equilibrated with buffer A (50 mM Tris-HCl, 150 mM sodium chloride, 0.5 mM oxtalamate, the EDTA-free cOmplete protease inhibitor cocktail (Roche Life Sciences), was recovered after a second round of reverse IMAC, concentrated 300 mM imidazole for column regeneration. The untagged IMPI containing untagged inhibitor was collected, whereas TEV, thioredoxin (produced in-house) at a peptidase:substrate ratio of 1:20 (w/w) in buffer A at room temperature to remove the fusion partner. After centrifugation (50,000 × g; 1 h; 4 °C) and elution in a gradient of 50–750 mM sodium chloride. Protein bound to the column was washed extensively using buffer D supplemented with 50 mM sodium chloride, and eluted in a gradient of 50–750 mM sodium chloride in the same buffer. The purified aureolysin was polished by SEC in a Superdex 75 10/300 column with buffer E (20 mM Tris-HCl, 150 mM sodium chloride, 10 mM calcium chloride, pH 7.8), and dialysed at 4 °C overnight against the same buffer. After centrifugation (50,000 × g; 1 h; 4 °C), the supernatant was loaded onto a 5–ML HiTrap Q FF anion exchange column (Cytiva) attached to an ÄKTA Pure 25 apparatus (Cytiva). The column was previously washed and equilibrated with buffer D, with or without 1 M sodium chloride. Protein bound to the column was washed extensively using buffer D supplemented with 50 mM sodium chloride, and eluted in a gradient of 50–750 mM sodium chloride in the same buffer. The purified aureolysin was polished by SEC in a Superdex 75 10/300 column with buffer E (20 mM Tris-HCl, 150 mM sodium chloride, 10 mM calcium chloride, 50 μM zinc chloride, pH 7.8).

Protein purity was assessed by SDS-PAGE on custom-made 14–20% glycine gels followed by staining with Coomassie Brilliant Blue (Sigma-Aldrich). Protein identities were confirmed by peptide mass fingerprinting (Suppl. Fig. 1) and N-terminal sequencing (Edman degradation) at the Protein Chemistry Service and the Proteomics Facility of the Centro de Investigaciones Biológicas (CIB-CSIC, Madrid, Spain). Ultrafiltration was carried out using Vivaspin 15 and Vivaspin 2 filter devices with HydroSart membranes and a 2-kDa cut-off (Sartorius Stedim Biotech). Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific) by comparison to a dilution series of bovine serum albumin.

Activity and inhibition assays — We tested the proteolytic and peptidolytic activity of aureolysin, thermolysin from B. thermoproteolyticus Rokko (Sigma-Aldrich), and ulinysin (produced according to [67,68]) at 37 °C in 100-μL reactions containing buffer F (100 mM Tris-HCl, 150 mM sodium chloride, 10 mM calcium chloride, 50 μM zinc chloride, pH 7.5) in an Infinite M200 microplate fluorometer (Tecan). As substrates, we used 10 μg/mL of the pig skin gelatin fluorescein conjugate from the DQ Gelatin EnzCheck.

Table 1

| Plasmid | Forward primer | Reverse primer | Template |
|---------|----------------|----------------|----------|
| pIMPI-T50N | CATATAAGATATAAAATAACTGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50Q | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50R | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50Y | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50Y + I55R | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50Y + I55R + I57F | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50Y + I55R | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |
| pIMPI-T50R | CATATAAGATAGAAAGACTTGGCCC | GGGCACATTATTTTATTCGTATAG | pIMPI-WT |

* This mutant was used as an intermediate to prepare T50Y and was not tested for activity. Only single nucleotides were exchanged in each reaction. For the double and triple mutants, a corresponding ancestral plasmid was used as the template.
Inhibition by wt-IMPI was measured using both substrates following the pre-incubation of the inhibitor (up to 200-fold molar excess) with 100 nM aureolysin, 10 nM thermolysin or 10 nM uliysin for 1 h at room temperature. Inhibition by the IMPI mutants (T50Q, T50R, T50Y, I54M, I55R, I55W, I55Y, I57F, I57Y, R58E, T50Y+I55R, and T50Y+I55R+I57F) was measured using FRET-4 following the pre-incubation of each mutant (up to 100-fold molar excess) with 50 nM aureolysin for 1 h at room temperature. Reactions were carried out at 37°C in buffer G (20 mM Tris HCl, 150 mM sodium chloride, pH 7.5) in triplicate and the residual proteolytic activity was measured for 3 h. The activity of the inhibitors in the absence of peptidase was monitored for the same duration as a negative control. To determine the relative activity of the IMPI mutants compared to the wild-type, initial cleavage velocities of the fluorogenic protein and peptide substrates, without (V0) and with (Vi) inhibitor, were determined from the slope of the linear range (R2 > 90%) of the fluorescence vs time curve, and (V0/Vi) was calculated using GRAPHDAP PRISM [69].

Complex formation and inhibitor cleavage detection — The complexes of aureolysin (at 100 µM) with wt-IMPI or the I57F-mutant were prepared by incubation in buffer H (50 mM Tris HCl, 150 mM sodium chloride, pH 8.0) at a 1:2.5 M ratio for 30 min at room temperature. The complex was then disrupted by SEC in a Superdex 75 10/300 GL column (GE Healthcare) previously equilibrated in buffer H. The same amounts of aureolysin and inhibitor were processed separately as controls. IMPI cleavage was analysed by SDS-PAGE as above and mass spectrometry in a MALDI-TOF Autoflex III instrument (Bruker). Each sample was desalted using a C18 ZipTip (Millipore), mixed at a 1:1 ratio (v/v) with a matrix solution of 10 mg/mL sinapic acid in 50% acetonitrile, and spotted onto the plate using the dried-droplet method. Mass spectra were acquired in linear-mode geometry. Internal calibration was performed by correction of the average mass of the respective non-treated IMPI control sample (wt-IMPI: 7927.6 Da; I57F-IMPI: 7967.1 Da).

Crystallisation and diffraction data collection — Crystallisation conditions were screened at the joint IRB/IBMB Automated Crystallography Platform using the sitting-drop vapor diffusion method. A Freedom EVO robot (Tecan) prepared screening solutions and dispensed them into the reservoir wells of 96/2-well MRC crystallisation plates (Innovadyne Technologies). A Phoenix/RE robot (Art Robbins) pipetted crystallisation nanodrops containing 100 nL of each protein and reservoir solution into the shallow wells, and plates were incubated in steady-temperature crystal farms (Bruker) at 4°C or 20°C. Optimal aureolysin crystals complexed with either wt-IMPI or I57F-IMPI formed at 20°C in solutions containing 5 mg/mL aureolysin and 2.9 mg/mL IMPI (peptidase:inhibitor molar ratio of 1:2.5) in 50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1.6 mM calcium chloride, 8.3 µM zinc chloride, which was mixed with reservoir solution consisting of 0.1 M Bis-Tris pH 5.5, 25% (w/v) PEG 3350 or 0.1 M Bis-Tris pH 6.0, 31% (w/v) PEG 2000 MME. Crystals were cryoprotected with reservoir solution

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**Fig. 1. Protein production and purification.** (A) Representative chromatogram and (B) SDS-PAGE analysis of the IMAC purification step of His6-thioredoxin-tagged wt-IMPI (expected molecular mass ~ 25 kDa). FT, flow-through; W, wash step; E, elution step. (C) Chromatogram and (D) SDS-PAGE analysis of the SEC purification step of tag-depleted wt-IMPI (~8 kDa), which migrated as a monomer (13.6 kL). (E) Representative chromatogram and (F) SDS-PAGE analysis of the anion-exchange chromatography purification step of aureolysin. (G) Chromatogram and (H) SDS-PAGE analysis of the SEC purification step of aureolysin. Despite the higher-than-expected molecular mass reported by SDS-PAGE (panels F,H), the protein is indeed mature aureolysin (expected mass ~ 33 kDa), as confirmed by N-terminal sequencing, peptide-mass fingerprinting (Suppl. Fig. 1), and the retention volume in calibrated SEC (panel G; 11.3 kL) corresponding to ~ 29 kDa. (I) 20% Glycine SDS-PAGE showing the purity of wt-IMPI and the 12 mutants (2–5 µg) analysed herein. All constructs behaved similarly to (A-D) during purification and yielded products of comparable purity and molecular mass.
plus 10% ethylene glycol, harvested using round LithoLoops of 0.04–0.1 mm (Molecular Dimensions), and flash-vitrified in liquid nitrogen for data collection. X-ray diffraction data were collected at 100 K on a Pilatus 6 M pixel detector (Dectris) at the XALOC beamline of the ALBA synchrotron (Cerdanyola, Catalonia, Spain) and on a Pilatus3 X 2 M detector (Dectris) at the ID23-2 beamline of the ESRF synchrotron (Grenoble, France). Diffraction data were processed with programs XDS [70] and XSCALE, and transformed with XDSCONV to MTZ-format for the PHENIX [71] and CCP4 [72] suites of programs. Statistics describing data collection and processing are provided in Table 2.

Structure solution and refinement — The structure of the complex of aureolysin and I$^{{19}}$F-IMPI was solved by molecular replacement using PHASER [73] on a dataset initially processed as space group P4$_1$2$_1$2 and at 2.05 Å resolution (Table 2), with one complex per asymmetric unit (a.u.). The coordinates of the protein part of the complex were from the Protein Data Bank (PDB) access code 1BQB [27] and wt-IMPI in a complex with unbound aureolysin (PDB 3SSB [62]) were used as searching models. These calculations yielded unique solutions for the peptidase and inhibitor dataset for the I$^{{57}}$F-IMPI complex with a higher resolution (1.60 Å).

The adequately rotated and translated molecules were refined using the REFINEMACS [79] considering twinning, as well as TLS and non-crystallographic symmetry (NCS) constraints. The final model included residues A$_{209}$–E$_{509}$, one zinc and three calcium ions of peptidase protomers A and C, as well as I$_{20}$–I$_{86}$ and I$_{20}$–P$_{84}$ of inhibitor moieties B and D, respectively, plus five ethylene glycol and 559 solvent molecules. Given that the structure of unbound aureolysin had originally been obtained before the gene sequence was available [27,49], it contained five erroneous residues at positions 354, 361, 479, 492, and 493, which were corrected in the final model of the complex.

The structure of the wt-IMPI complex with aureolysin was solved at a resolution of 1.85 Å by Fourier synthesis after rigid-body refinement using the coordinates of the refined mutant complex structure. Model completion and refinement were carried out as described above. The final model comprised residues A$_{209}$–E$_{509}$ and A$_{209}$–V$_{509}$ of peptidase molecules A and C, plus one zinc and three calcium ions each, as well as I$_{20}$–I$_{86}$ and I$_{20}$–K$_{85}$ of inhibitor moieties B and D, respectively. Two diethylene glycol, three ethylene glycol, and 709 solvent molecules completed the model. Table 2 provides essential statistics on the final refined models, which were validated using the wwPDB validation service (https://validate.rcsb.org/wwpdb/validservice) and deposited at www.pdb.org (access codes 7SKL and 7SKM).

Miscellaneous — Structure superpositions were calculated with SSM [80] in COOT. Figures were prepared using CHIMERA [81]. Protein interfaces and intermolecular interactions were analysed using PDREPISA [82] (www.ebi.ac.uk/pdbe/pisa) and verified by visual inspection. The interacting surface of a complex was taken as half the sum of the buried surface areas of either molecule.
3. Results and discussion

Assessment of wild-type IMPI as an aureolysin inhibitor and initial protein redesign — Wild-type IMPI was expressed in E. coli and recovered in a highly pure form (Fig. 1A–D; Suppl. Fig. 1). To assess its effect on aureolysin, which in turn was purified to homogeneity from cultures of S. aureus (Fig. 1E–H; Suppl. Fig. 1), the inhibitor was tested at molar ratios of 1:1 to 1:200 using a fluorogenic protein (Fig. 2A) and a fluorogenic peptide (Fig. 2B). We also tested thermolysin (the archetypal M4 family MP) and ululysin, a metzincin MP from the pappalyisin family (MEROPS M43B; [67,68]) as controls. Thermolysin was efficiently inhibited as expected, whereas ululysin was not inhibited at all, in agreement with IMPI being a specific inhibitor of M4 family MPs. Aureolysin was also inhibited in a dose-dependent manner, particularly when using the peptide substrate, although not to the same extent as thermolysin.

We superposed the structure of unbound aureolysin [27] onto thermolysin in a complex with wt-IMPI [62] and hypothesised that replacing I57 (whose side chain interacts with the MP, see below) with a bulkier residue such as phenylalanine might achieve stronger inhibition. Accordingly, we produced the mutant I57F-IMPI as described above for wt-IMPI (Fig. 1I) and used it for further analysis.

Overall structure of the IMPI–aureolysin complex — We crystallised I57F-IMPI and wt-IMPI in complexes with aureolysin (Fig. 3A) and used molecular replacement to solve their tetragonal (P41) crystal structures, which contained two complexes per a.u. Structural solution and refinement (to 1.60 and 1.85 Å, respectively) was hindered by the presence of merohedral twinning in both crystals, with twinning fractions of 0.536 and 0.490, respectively (Table S1). Even so, the structures were refined to final free R-free values of 0.188 and 0.219, respectively, which are considered accurate. This was confirmed by the final Fourier maps (Fig. 3E). The two structures were practically indistinguishable upon superposition, so the following discussion focuses on the I57F-IMPI complex (protomers A and B) if not otherwise stated.

The structure of wt-IMPI has been reported in a complex with thermolysin [62]. Briefly, it has a spearhead shape (Fig. 3B), whose tip contains a “reactive-site bond” (RBS; N312–I/F57) within a RCL (C32–C53) that precedes the upper-rim strand and protrudes from the surface above the cleft (Fig. 3C). The C-terminal subdomain (CSD; N364–E509; Fig. 3C) starts with the characteristic “glutamate helix” of gluzincins [19,20], which contains the third zinc-binding protein ligand (E376; Fig. 3E). It is followed by a long “irregular segment” (D388–G434) that shapes the bottom of the active-site cleft on its primed side, including the hydrophobic S1 pocket. This pocket confers substrate specificity upon aureolysin and other M4 family MPs, as well as most other MP families [53]. Moreover, the irregular segment embraces three calcium-binding sites, which stabilise the structure [28]. The removal of these ions using chelators therefore causes irreversible inactivation [28,86]. The CSD also contains a C-terminal four-helix bundle arranged as a Greek-key motif. Remarkably, the aureolysin CSD lacks the conspicuous β-ribbon that protrudes from the last turn of the first of these α-helices in thermolysin.

In the complex, I57F-IMPI inserts like a wedge into the active-site cleft of the peptidase (Fig. 3D) and interacts via interfaces of 865 and 849 Å² (ΔΔG = –5.2 and –4.5 kcal/mol [82]) in complexes A/B and C/D, respectively. This involves 24 hydrogen bonds and salt bridges, plus two metalorganic bonds, as well as hydrophobic interactions between five inhibitor and 10 peptide residues (Table 3). The main participating elements are the RCL and scaffold loop of the inhibitor, as well as the flap, upper-rim strand, S1-pocket shaping residues, and the initial and final stretches of the irregular segment. Diverging from the thermolysin complex, superposition of the aureolysin complexes with wt-IMPI and I57F-IMPI revealed a much smaller spread in the relative orientation between inhibitor and peptidase. The maximum deviation at the cleft-distal site of the inhibitor was ~4°/1.8 Å across the four

![Fig. 2. Inhibitory activity of wild-type IMPI. (A) Residual fractional activity as V_i/V_0 relative to the activity in the absence of inhibitor of (left) 10 nM ululysin, (middle) 100 nM aureolysin, and (right) 10 nM thermolysin after incubation with wt-IMPI at several molar ratios using the DQ gelatin substrate. (B) As above, but using the internally quenched fluorescent FRET-4 peptide as the substrate.](image-url)
Stratex, blocking S4 IMPRCL runs across the peptidase cleft in the direction of the sub-

teinase [27]. Aureolysin therefore does not appear to undergo the closing hinge motion when binding ligands or substrates, in contrast to other M4 family MPs.

P. aeruginosa elastase and Bacillus cereus neutral proteinase [27]. Aureolysin therefore does not appear to undergo the closing hinge motion when binding ligands or substrates, in contrast to other M4 family MPs.

IMPI inhibits aureolysin via the standard mechanism — The IMPI RCL runs across the peptidase cleft in the direction of the substrate, blocking S4–S1’ with residues P13–P1 (Fig. 3H). Remarkably, the RSB was cleaved in the crystals [Fig. 3E], which was verified in vitro by incubating both wt-IMPI and I37F-IMPI with aureolysin. Indeed, both forms were cleaved at N56–I/F57 (Fig. 3F, G). This feature causes the terminal carboxylate oxygen of the P1 residue, N36OT, to bind the catalytic zinc and contribute to a distorted tetrahedral coordination sphere together with protein ligands H352N, H356N2, and E376O2 (all 2.02–2.11 Å apart in the various structures). N56OT replaces the two solvent molecules found in the unbound structure [27] and further contacts H436N2 (3.08–3.15 Å), which is equivalent to H231, of thermolysin (thermolysin residues are shown in italics with subscript numbers for clarity). Together with Y376, equivalent to Y157 in aureolysin, this residue helps to stabilise the tetrahedral reaction intermediate [85]. Moreover, the other carboxylate oxygen of N36 is very close to the general base/acid glutamate (N36O–E353O, 2.60–2.67 Å), indicating that one of them must be protonated. On the primed side of the cleft, P1 residue I/F17 is bound via its α-amino group to E353O2 (2.90–3.01 Å) and the upper-rim main-chain carbonyl

Fig. 3. Structure of the IMPI–aureolysin complex. (A) Tetragonal protein crystals of the aureolysin–wt-IMPI (left) and aureolysin–I37F-IMPI complexes (right). (B) Ribbon-type plot of P13F-IMPI depicting the four β-strands (β1–β4) and the single helix (α1) of the structure, as well as the five disulfide bridges (with numbered cysteine residues). The scaffold loop is shown in blue, and the reactive-centre loop (RCL) is shown in pink with numbered residues (sticks). The cleaved reactive-site bond (RSB), N-terminus, and C-terminus are labelled. Hydrogen bond N56O–I57N is needed to maintain the position of the P1 residue in place. (C) Ribbon-type plot of the complex between P13F-IMPI (green ribbon, disulfide bonds as yellow sticks) and aureolysin (pale gold ribbon, catalytic zinc and structural calcium cations shown as magenta and red spheres, respectively) viewed along the active-site cleft (vertically rotated 90°) from the traditional “standard orientation” of MPs [53]). The side chains of the zinc-binding MP residues and the general/base acid glutamate are further shown as sticks for reference (carbons in salmon). The N-terminus and C-terminus are labelled, the characteristic flap is in purple, and the NSD and CSD of the peptidase are indicated. (D) Rotated view of (C). (F) Close-up in cross-eye stereo showing the RCL and scaffold loop of P13F-IMPI (green carbons) and the zinc site of aureolysin (carbons in salmon) superposed with the final 1.60-Å (2mFobs–DFcalc)-type Fourier map as a semi-transparent surface contoured at 1 σ in a similar view to (D). The RSB is cleaved, selective inhibitor and MP residues are numbered in red and blue, respectively. Hydrogen bond N56O–I57N is shown as a dashed line. (F) In vitro proof that binding and inhibition of aureolysin by wt- and I37F-IMPI involves the cleavage of the inhibitor at the RSB (N56–I57) within the RCL as shown by SDS-PAGE analysis of the respective SEC fractions. (G) Mass spectra showing analysis of the cleavage of (top) intact wt-IMPI (blue spectrum; 7933.1 Da) giving rise to the cleaved inhibitor (red spectrum; 7676.3 Da) and (bottom) intact I37F-IMPI (blue spectrum; 7967.1 Da) to yield the cleaved inhibitor (red spectrum; 7710.0 Da). Incubation of both intact species with aureolysin leads to the removal of the N-terminal tag-segment G–M (–275 Da) and the addition of a water molecule (+18 Da) due to RSB cleavage. For wt-IMPI, a small fraction of tag-depleted noncleaved inhibitor was detected (7658.3 Da). (H) Close-up in stereo of (D), further rotated 25° downwards and 25° leftwards, giving insight into the interactions between P13F-IMPI (sticks with green carbons, residue numbers in red) and aureolysin (sticks with carbons in salmon, residue numbers in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

complexes of the two structures, compared to ~ 10°/4.8 Å for the two thermolysin complexes in the a.u. [62].

Finally, superposition of IMPI-bound aureolysin with the unbound structure [27] revealed negligible differences between the NTS and CTS. This contrasts with thermolysin, where a 5° relative rotation of the two subdomains distinguishes between the unbound and bound forms [87]. Similar relative motion was proposed for P. aeruginosa elastase and Bacillus cereus neutral proteinase [27]. Aureolysin therefore does not appear to undergo the closing hinge motion when binding ligands or substrates, in contrast to other M4 family MPs.
of A$_{322}$ (3.14–3.27 Å) as well as the side-chain carboxamide of N$_{322}$ (3.09–3.25 Å; Fig. 3E,H).

The inhibition mode described above agrees with the “standard mechanism” or “canonical mechanism” of peptidase inhibition [88,89]. Remarkably, in standard-mechanism inhibitors (which mostly target serine endopeptidases), the RSB is cleaved very slowly because the cleavage reaction is kinetically unfavourable, so the intact complexes have half-lives of several years [90]. This has been verified by many crystal structures with intact RSBs [91]. In contrast, IMPI represents a unique case of a standard-mechanism MP inhibitor occurring as a cleaved inhibitor, first in its thermolysin complex [62] and now here with aureolysin, whose 69-residue structure is kept rigid through five disulfide bonds that are evenly distributed across the structure.

Finally, in the aureolysin complexes, the cleaved RSB is poised for rejoining, which is another functional requisite of the standard mechanism [91]. This is indicated by the proximity and orientation of the $\alpha$-amino group of I/F$_{57}$ relative to the carboxylate carbon of N$_{56}$, which are ideally situated for a nucleophilic attack. Indeed, the angle I/F$_{57}$N–N$_{56}$C–N$_{56}$OT, where N$_{56}$OT is the oxygen that is not bound to the general base/acid glutamate, is $/C24110/C176$ on average over all four I$_{57}$F-IMPI and wt-IMPI complexes, thus in good agreement with the value postulated for a productive Bürgi-Dunitz geometrical reaction coordinate (105 ± 5$^\circ$) [92]). This is supported by the ability of cleaved wt-IMPI to rejoin in vitro following the addition of catalytic amounts of thermolysin [62].

Table 3

Interactions at the I$_{57}$F-IMPI–aureolysin interface.

| Hydrogen bonds/salt bridges (<3.7 Å) | Hydrophobic interactions (<4 Å) |
|--------------------------------------|----------------------------------|
| Y$_{31}$O–K$_{430}$N 3.10/2.79 Å     |                                  |
| E$_{32}$O–D$_{341}$N 2.75/2.82 Å    |                                  |
| A$_{35}$O–Q$_{317}$N 2.72/2.76 Å    |                                  |
| D$_{38}$N–Q$_{317}$O 2.72/2.84 Å    |                                  |
| A$_{38}$O–Q$_{317}$N 2.72/2.84 Å    |                                  |
| F$_{57}$N–N$_{322}$O 3.14/3.27 Å    |                                  |
| F$_{57}$N–A$_{323}$O 3.27/3.14 Å    |                                  |
| F$_{57}$N–E$_{353}$O 2.90/3.01 Å    |                                  |
| F$_{57}$N–E$_{353}$O 2.90/3.01 Å    |                                  |
| F$_{57}$O–R$_{408}$N 2.83/2.75 Å    |                                  |
| F$_{57}$O–R$_{408}$N 2.83/2.75 Å    |                                  |
| K$_{58}$N–Q$_{317}$O 2.51/3.15 Å    |                                  |
| R$_{58}$N–Q$_{317}$O 2.51/3.15 Å    |                                  |
| I$_{58}$N–W$_{325}$O 2.85/2.76 Å    |                                  |
| I$_{58}$O–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |
| I$_{58}$N–W$_{325}$N 2.74/2.78 Å    |                                  |

The first residue/atom belongs to IMPI, the second to aureolysin. The two values for each bond correspond to complexes between protomers A/B and C/D, respectively.
Redesign of IMPI — Based on the IMPI—aureolysin crystal structures described above, we identified positions 50, 54, 55, 57 and 58 of the RCL as ideal for mutagenesis and constructed 11 single, double and triple point mutants in addition to the wt-IMPI and I127F-IMPI variants (T50Q, T50R, T50Y, I54M, I55R, I55W, I55Y, I57V, R58E, T50Y + I55R, and T50Y + I55R + I57F). All variants were produced and purified as efficiently as described above for wt-IMPI (Fig. 1I), and were compared to wt-IMPI for their ability to inhibit aureolysin at molar ratios of 1:1 to 1:100 using the fluorogenic peptide FRET-4 as the substrate (Fig. 4AB). R58E did not affect peptide activity. We tested the mutant with thermolysin, which revealed ~200-fold weaker inhibition than the wild type (Suppl. Fig. 2). We thus conclude that the mutant was properly folded, as suggested by its behaviour during purification, but functionally impaired and thus unable to block thermolysins. The rest of the cohort of mutants achieved the concentration-dependent inhibition of aureolysin. They could be assigned to two groups, one similar to the wild type, with residual activities of 3–8% at the highest molar ratio (Fig. 4B), whereas the others showing weaker inhibition, with residual activities of 17–58% (Fig. 4B). The derived IC50 values enabled us to estimate Ki values of 346–644 nM for the first group and 1220–4520 nM for the second group (Fig. 4B). Notably, mutant I127F (from the initial stage of the project, see above) achieved the highest inhibition among all variants tested (Ki = 346 nM) and would thus provide a suitable lead for further development.

Corollary — Aureolysin plays multiple roles during S. aureus infections and is a promising target for the development of novel antimicrobials. We tested the M4-specific inhibitor IMPI, and found that it inhibited the peptidase using the standard mechanism, best described for serine endopeptidases, based on the analysis of crystal structures. We therefore designed a cohort of point mutants, with I127F emerging as the strongest inhibitor. This is, to our knowledge, the first report of a TP candidate that can inhibit one of the major proteolytic virulence factors of S. aureus. The only other protein-based inhibitor with this ability is the general pan-peptidase inhibitor σ2-macroglobulin, which has a molecular mass of ~720 kDa and a broad spectrum of targets, making it unsuitable for therapeutic applications. Cell-based and disease challenge studies are now required to confirm the potential of I127F-IMPI as a TP for the treatment of S. aureus infections.

Author contributions

F.X.G.R. and A.V. conceived, supervised, and funded the project; S.R.M. produced and purified all proteins, prepared the mutants, performed in vitro studies with U.E. and P.C., analysed kinetics and mass spectrometry data with U.E., and crystallised proteins with assistance from U.E. and T.G.; S.R.M., U.E. and A.R.-B. collected diffraction data, and U.E. performed initial data analysis; E.M. performed biocomputational calculations; F.X.G.R. solved and refined crystal structures; and F.X.G.R. and A.V. wrote the manuscript with contributions from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.01.001.

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