A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

GDCh

www.angewandte.org

Accepted Article

Title: A Chemical Disruptor of the ClpX Chaperone Complex Attenuates Multiresistant Staphylococcus aureus Virulence

Authors: Christian Fetzer, Vadim S Korotkov, Robert Thänert, Kyu Myung Lee, Martin Neuenschwander, Jens Peter von Kries, Eva Medina, and Stephan Axel Sieber

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201708454
Angew. Chem. 10.1002/ange.201708454

Link to VoR: http://dx.doi.org/10.1002/anie.201708454
http://dx.doi.org/10.1002/ange.201708454

WILEY-VCH

10.1002/anie.201708454

WILEY-VCH

COMMUNICATION

A Chemical Disruptor of the ClpX Chaperone Complex Attenuates Multiresistant *Staphylococcus aureus* Virulence

Christian Fetzer[a], Vadim S. Korotkov[a], Robert Thänert[b], Kyu Myung Lee[a], Martin Neuenschwander[c], Jens Peter von Kries[c], Eva Medina[b] and Stephan A. Sieber*[a]

Abstract: *The Staphylococcus aureus ClpXP protease is an important regulator of cell homeostasis and virulence. Here we utilize a high-throughput screen against the ClpXP complex and identify a specific inhibitor of the ClpX chaperone that disrupts its oligomeric state. Synthesis of 34 derivatives revealed that the molecular scaffold is restrictive for diversification with only minor changes tolerated. Subsequent analysis of the most active compound revealed strong attenuation of S. aureus toxin production which was quantified via a customized MS-based assay platform. Transcriptome and whole proteome studies further confirmed the global reduction of virulence and unraveled characteristic signatures of protein expression in compound treated cells. Although these partially matched the pattern of ClpX knockout cells, further depletion of toxins was observed leading to the intriguing perspective that additional virulence pathways may be directly or indirectly addressed by the small molecule.*

Staphylococcus aureus is a gram-positive pathogen responsible for devastating infections of e.g. lungs, skin and bones.[1] A diverse arsenal of virulence factors including several proteases and cytolysins facilitates the establishment of infection by enabling the bacterium to thwart the immune response and survive within the host.[2] The treatment and prevention of staphylococcal infections with classical antibiotics has become challenging due to the increasing prevalence of multiresistant *S. aureus* strains such as methicillin-resistant *S. aureus* (MRSA). Thus, alternative strategies that target bacterial virulence rather than viability have been proposed.[3] Caseinolytic protease (ClpP) was shown to play a crucial role in toxin expression[4] and its genetic deletion resulted in a dramatic reduction in hemolysin alpha (Hla) production.[5] ClpP associates with chaperones such as ClpX that recognize and unfold substrate proteins.[6] Accordingly, also ClpX represents a crucial target for anti-virulence strategies.[5] While only little is known about chemical inhibition of ClpX,[7] several specific ClpP inhibitors, foremost beta-lactones, were shown to attenuate *S. aureus* virulence *in vitro* and *in vivo*.[8] However, the facile ester bond limited pharmacological applications and prompted the search for alternative inhibitor scaffolds. In fact, reversible oxazole inhibitors showed enhanced stability and potency but were rapidly ejected from the ClpP active site upon chaperone binding.[9] Hence, we here performed a high-throughput screen (HTS) against the whole ClpXP complex and identified one potent hit molecule that inhibits proteolysis by dissociation of ClpXP protein interactions and globally reduces virulence of *S. aureus* and MRSA.

For screening of ClpXP inhibitors, an established assay based on the degradation of green fluorescent protein (GFP) equipped with a SsrA peptide-tag was used.[10] This native peptide sequence is recognized by ClpX, which unfolds the tagged GFP under ATP consumption. Subsequently, the linear peptide chain is digested within the proteolytic ClpP barrel resulting in a loss of fluorescence signal. Putative inhibitors of proteolysis can either target ClpP, ClpX, the interaction between these two components, or the ATP regeneration system requiring careful validation of hits in secondary assays. Given the high concentration of ATP in the assay, identification of ATP binding site competitors in ClpX was unlikely, while inhibitors of the GFP-SsrA substrate channel (low µM K_M in *E. coli*)[10] could be possible. We adapted the fluorescence assay for HTS (see SI for details) and screened about 40.000 compounds from the FMP library (Z-factor 0.69, Figure 1A). 332 molecules were identified as primary hits deviating from the normal distribution by three standard deviations (z-score > 3). To select the most potent compounds and exclude inhibitors of the ATP regenerating system, IC_{50} values were determined and molecules assayed against creatine kinase in a secondary screen (Figure S1). Six compounds with IC_{50} values ranging between 0.6 and 3.1 µM were selected for a closer inspection of their mode of action.

For this we performed several assays focusing on either ClpP, ClpX or the ClpXP complex. Interestingly, none of the hit compounds inhibited ClpP peptidase activity up to a concentration of 25 µM (25-fold excess), suggesting that these molecules exhibit a novel inhibitory mechanism (Figure S2). Compounds **334** and **336**, which encompass a similar structural core motif (Figure 1B), blocked ClpX ATPase activity with an IC_{50} of 0.8 and 1.8 µM, respectively, while all other compounds were largely inactive (Figure S3). Inhibition of chaperone activity is an intriguing finding since specific ClpX inhibitors have not been reported so far. Both hits did not alter ClpCP proteolysis demonstrating selectivity solely for ClpX (Figure S4).

We further focused on compound **334** as the most potent ClpX inhibitor and analyzed its mechanism of action. First, the **334** structure does not exhibit any obvious reactive electrophilic moieties and accordingly no covalent modification of ClpX was obtained by intact-protein mass spectrometry (Figure S5). Second, ClpX hexamer stability was evaluated in presence and

[a] C. Fetzer, V. S. Korotkov, K. M. Lee, S. A. Sieber
Center for Integrated Protein Science at the Department of Chemistry, Technische Universität München
Lichtenbergstrasse 4, 85747 Garching (Germany)
E-mail: stephan.sieber@tum.de

[b] R. Thänert, E. Medina
Infection Immunology Research Group, Helmholtz Centre for Infection Research
Inhoffenstrasse 7, 38124 Braunschweig (Germany)

[c] M. Neuenschwander, J. P. von Kries
Leibniz-Forschungsinstitut für Molekulare Pharmakologie
Robert-Roessle-Strasse 10, 13125 Berlin (Germany)

* To whom correspondence should be addressed: S.A.S.: stephan.sieber@tum.de

Supporting information for this article is available on the WWW under http://dx.doi.org/

10.1002/anie.201708454

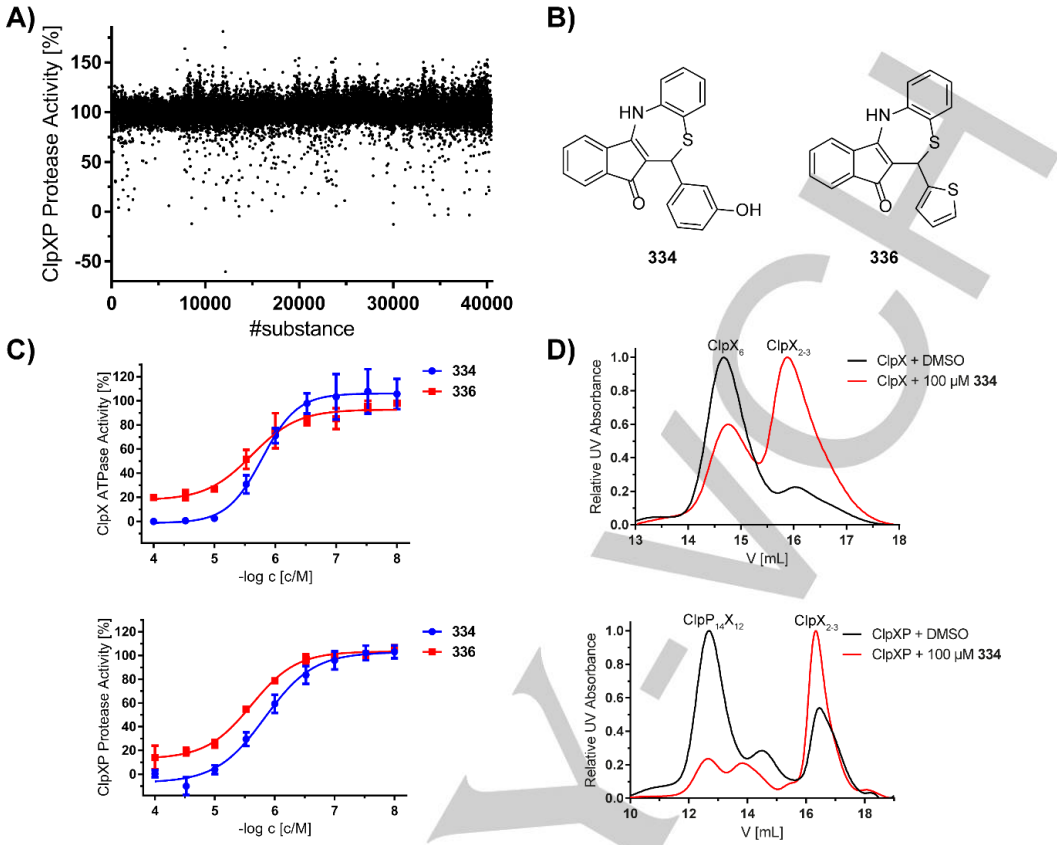
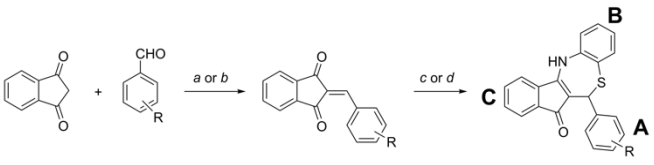


Figure 1. HTS of 40,000 compounds revealed potent inhibitors of ClpX and ClpXP. A) Initial screen with 10 µM compound concentration yielded 332 compounds for further validation. B) Two potent compounds, **334** and **336**, share the same structural motif. C) **334** and **336** are potent inhibitors of both ClpX ATPase activity and ClpXP protease activity (mean ± standard deviation of at least three experiments). D) Size-exclusion chromatography experiments show the disruption of ClpX-hexamer and ClpXP-complex upon treatment with 100 µM **334**.

absence of **334** via analytical size-exclusion chromatography (Figure 1D). Importantly, a dramatic disruption of the oligomeric state to dimeric/trimeric species was observed upon compound addition. Third, even the whole ClpXP proteolytic complex collapsed in response to **334** binding (Figure 1D). Fourth, **334**-induced deoligomerization of ClpX was associated with a decrease in the melting temperature of 2.8 K as obtained in thermal-shift assays indicating destabilization of the complex (Figure S6). No disruption of protein-protein interactions could be observed with the other four hit compounds implying a distinctive mode of action.

To dissect the molecular prerequisites for **334** inhibitory activity we prepared 31 derivatives varying in their aromatic ring substitutions. The synthetic strategy was initiated by condensation of indan-1,3-dione with the corresponding benzaldehydes. The desired dihydrothiazepines were obtained by the reaction of 2-benzylideneindan-1,3-diones with 2-aminothiophenol (Scheme 1).

Introduction of a methoxy substituent at 5 position in the upper B-benzene ring (**345**) almost completely abolished inhibition of ClpXP suggesting that this site is less suited for structural modifications (Figure 2A). An exchange of the lower phenol ring A by thiophene (**336**) retained potency while a replacement by pyridine (**352**) resulted in a significantly increased IC_{50} value. Interestingly, the phenol ring turned out to be amenable for the introduction of additional hydroxy- (**347**), bromo- (**358**) or methoxy- (**344**) groups. However, positioning of the phenolic hydroxy-group in meta (parent **334**) and para (**343**) was crucial for activity while ortho (**351**) resulted in a significant drop of potency. Other substituents at meta-position including fluorine (**348**), methoxy (**341**), amino (**359**) and hydroxymethyl (**367**) showed reduced activity similar to the unsubstituted benzene (**340**). Together with the observation that bulky substituents (**365**, **350**) were not tolerated, we conclude, that the A-benzene ring is like the B-ring crucial for enzyme binding. A certain degree of structural flexibility was observed for the C-ring where chlorine (**366**) and alkynyl (**356**) substituents were tolerated. Oxidation of the thioether to a sulfone (e.g. **355**) resulted in a tenfold drop of potency. Of note, some compounds, although exhibiting comparable IC_{50} values, largely varied in the extent of maximum inhibition ranging from 100 % (e.g. **334**) to 8 % (e.g. **375**)



Scheme 1. Synthesis of dihydrothiazepines. Reagents and conditions: *a)* HOAc, NaOAc, reflux, 3 h; *b) L*-proline, MeOH, r. t., 16 h; *c)* 2-aminothiophenol, EtOH, r. t., 18 h; *d)* 2-aminothiophenol, *i*PrOH/HOAc, r. t., 18 h.

10.1002/anie.201708454

(Figure 2B). This phenomenon could be caused by varying degrees of conformational changes induced by the compound. Especially a hydroxy group in meta position seems to be a crucial trigger for full inhibition, however, the exact mechanism is not yet understood.

These initial structure-activity relationship (SAR) studies revealed flexibility for compound functionalization and guided the design of probes for *in situ* target validation via affinity-based protein profiling (AfBPP).[11] However, due to UV-lability of the core structure and SAR restrictions for attaching the photoreactive moiety, we concluded that development of a functional probe is, despite massive synthetic efforts, not feasible (see SI for detailed discussion).

Alternatively, we utilized a multidisciplinary strategy based on toxin secretion assays, RNA sequencing and full proteome analysis via LC-MS/MS to characterize the phenotypic effects of **334** and establish putative links with ClpX. Seminal studies by Frees *et al.* already demonstrated a global reduction of toxin secretion in a *S. aureus clpX* knockout strain.[5] One predominant trait of *S. aureus* virulence is the secretion of hemolysins. We thus treated *S. aureus* with various concentrations of **334** overnight and applied the bacterial supernatants to sheep-blood agar plates to assess the level of hemolysin production. As expected, a concentration dependent reduction of hemolysis activity with an IC_{50} of 3 µM was observed (Figure S7). Moreover, overall proteolysis, an additional hallmark of staphylococcal virulence,

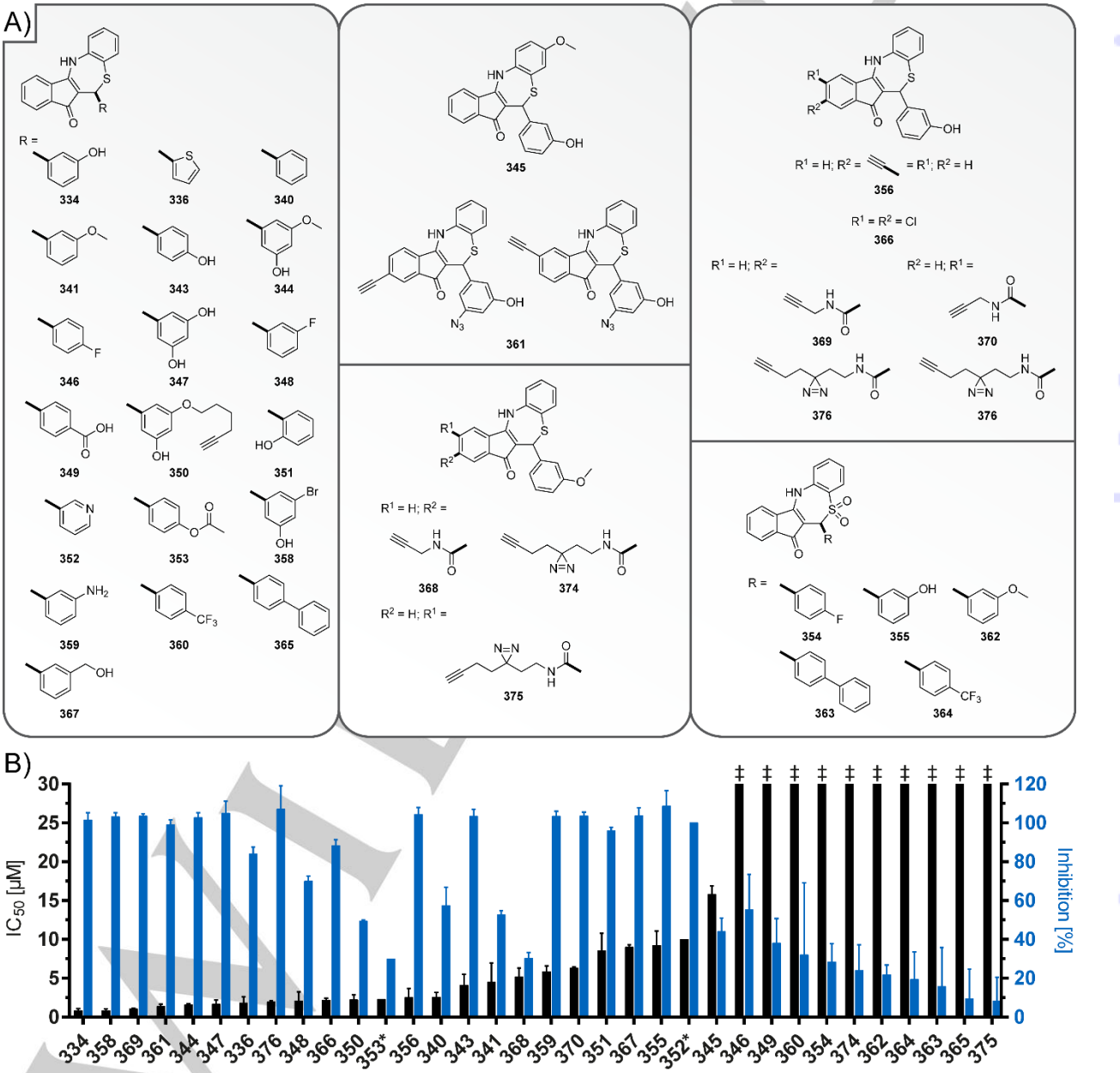
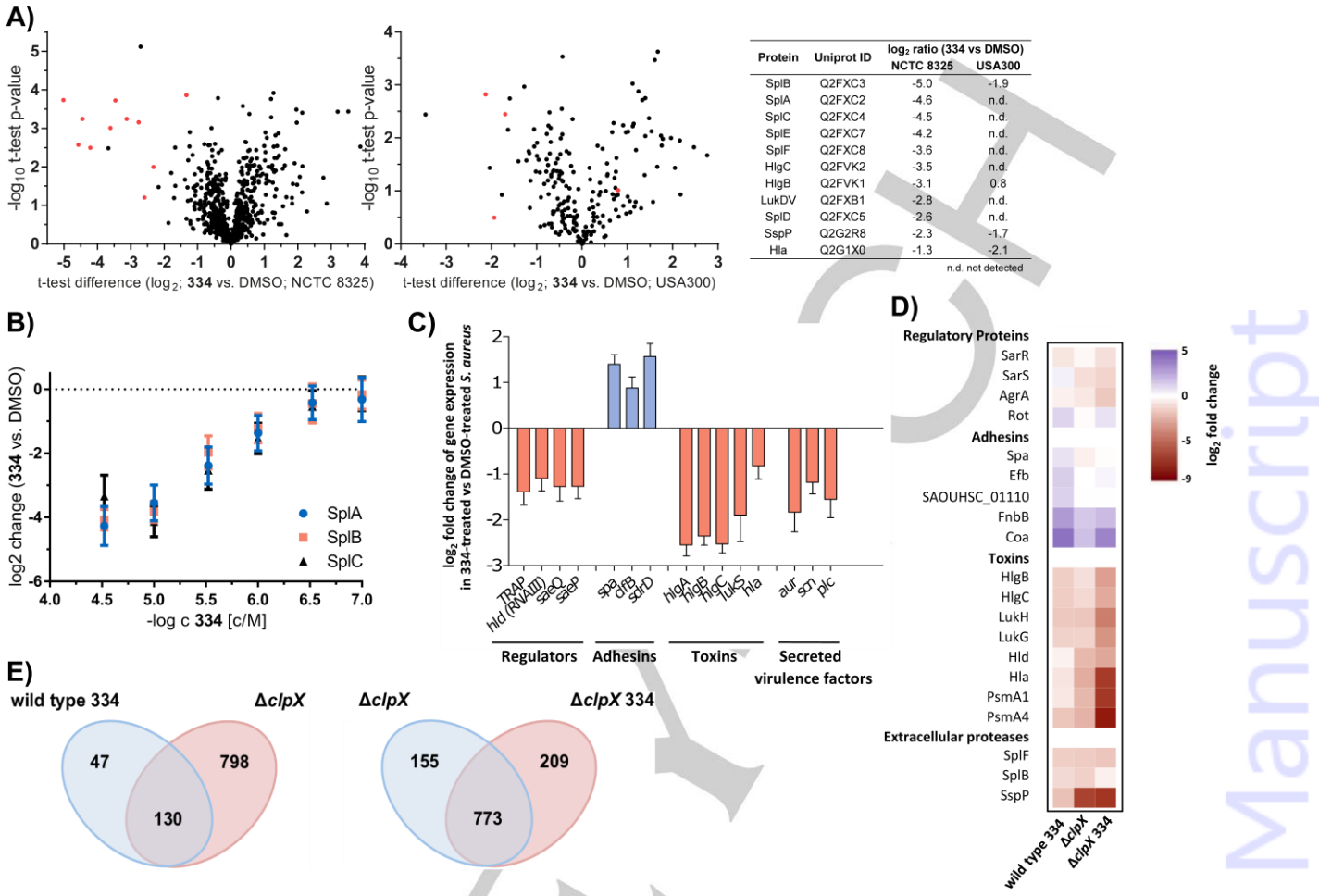


Figure 2. Structure-activity relationship studies of **334** analogues in ClpXP-protease assay. A) Synthesized derivatives of compound **334**. B) Inhibition data of all compounds in the ClpXP-assay. Shown are IC_{50} values (black bars) and the degree of inhibition (blue bars). * only tested in the HTS validation; ‡ compounds exhibited an IC_{50} > 50 µM. Mean ± standard deviation from at least three experiments.

10.1002/anie.201708454

WILEY-VCH



Protein	Uniprot ID	log2 ratio (334 vs DMSO) NCTC 8325	USA300
SplB	Q2FXC3	-5.0	-1.9
SplA	Q2FXC2	-4.6	n.d.
SplC	Q2FXC4	-4.5	n.d.
SplE	Q2FXC7	-4.2	n.d.
SplF	Q2FXC8	-3.6	n.d.
HlgC	Q2FVK2	-3.5	n.d.
HlgB	Q2FVK1	-3.1	0.8
LukDV	Q2FXB1	-2.8	n.d.
SplD	Q2FXC5	-2.6	n.d.
SspP	Q2G2R8	-2.3	-1.7
Hla	Q2G1X0	-1.3	-2.1

n.d. not detected

Figure 3. Treatment with **334** reduces the transcription and synthesis of toxins and proteases by *S. aureus*. A) Secretome analysis of **334** (10 µM) treated *S. aureus* NCTC 8325 and USA300 reveals lower levels of secreted toxins in comparison to DMSO treated cells. Red dots in volcano plots represent toxins listed in table. B) Titration of **334** in MS experiments leads to concentration dependent LFQ detection of toxin abundances in NCTC 8325. Mean ± standard deviation from three experiments. C) Changes in the expression levels of selected genes encoding virulence factors or regulators by *S. aureus* wt in response to **334** treatment. Mean ± standard error from three experiments. D) Heatmap showing the changes in protein abundance induced by **334** treatment in wt *S. aureus* in comparison with DMSO control (left lane), Δ*clpX* in comparison with *S. aureus* in DMSO control,(middle lane) and **334**-treated Δ*clpX* compared to *S. aureus* in DMSO control (right lane). A red-blue color scale depicts protein expression levels (blue: high, red: low). E) Venn diagram showing the numbers of proteins with changes in abundance that are shared or unique between **334**-treated *S. aureus* wt and *S. aureus* Δ*clpX*, or between DMSO-treated *S. aureus* Δ*clpX* and **334**-treated *S. aureus* Δ*clpX*.

was significantly reduced (Figure S7). To globally monitor the expression of virulence proteins that are affected by **334**, we established an MS-based whole secretome analysis platform. *S. aureus* NCTC 8325 as well as MRSA strain USA300 were incubated overnight in the presence of 10 µM **334** or DMSO. The bacterial supernatant was collected, proteins digested and modified for quantitative analysis. Importantly, the visualization of **334**/DMSO protein ratios of NCTC 8325 and MRSA in the respective volcano plots (Figure 3A) shows a dramatic and global down-regulation of major virulence factors including toxins (hemolysins alpha and gamma, leukotoxin) and diverse proteases (serine proteases A-F, staphopain). In addition, our novel MS-based virulence assay allowed the individual determination of IC_{50} values for each detected toxin. For this purpose, LC-MS/MS analysis was performed via label free quantification at various **334** concentrations. All IC_{50} values are in the low µM range corroborating the results of the hemolysis assay (Figure 3B and S8).

To gain more detailed insights into the inhibitory activity of **334,** we analyzed changes in the global gene expression of *S. aureus* induced by treatment with **334** using RNA-seq. Strikingly, the reduced expression of genes encoding toxins (*hla*, *hlgA/B/C*, *hld*, *lukS*) and the proteases (*aur*) matched the secretome data (Figure 3C) and are in accordance with previous studies reporting reduced extracellular virulence factor expression in absence of ClpX.[5] Notably, **334** treatment had a remarkable impact on the expression of important regulatory systems such as RNAIII, Sae, and TRAP, which control the expression of a large number of virulence factors including several toxins and adhesins (Figure 3C). The secretome and transcriptome data demonstrate the significant impact of **334** on *S. aureus* expression and production of virulence factors.

To determine, if this effect was mediated by the **334**-targeting of ClpX, whole proteome analysis was performed on *S. aureus* wt after treatment with **334** or DMSO as well as on a *S. aureus* ClpX knock out (Δ*clpX*)[5] after incubation with **334** or DMSO. Treatment

of wt *S. aureus* with **334** resulted in reduced expression of extracellular toxins and proteases and in an increased production of adhesins (Figure 3D). The effect of **334** on the level of virulence factor expression was largely comparable to that observed after genetic deletion of ClpX (Figure 3D). Therefore, the significant overlap of 130 proteins with changes in overall abundance (of which 84 are regulated in the same direction) induced by **334** with those induced by the genetic deletion of ClpX suggested, at least in part, an inhibitory effect on ClpX (Figure 3E). Interestingly, **334** also significantly affected the expression of virulence factors and other proteins in the Δ*clpX* mutant (Figure 3D and 3E). These findings indicate that **334** could address additional targets that further enhance the inhibitory effect on the production of extracellular virulence factors by ClpX. Due to the restrictions of **334** for application in chemical proteomics as discussed above, assessing the direct effect of the compound on key regulators within the agr system will constitute the most straightforward way for the identification of these putative targets in future studies.

In conclusion, we introduced the first reversible inhibitor of ClpX, which retained activity against the whole proteolytic ClpXP complex, a superior trait compared to previous reversible ClpP binders. In addition, global transcriptome and proteome analysis pointed towards targeting of ClpXP also in living cells. Accordingly, a reduction of virulence expression was detected via classical assays and more comprehensively with a MS platform allowing reliable *in situ* toxin monitoring. Given the susceptibility of the Δ*clpX* mutant strain towards **334** we anticipate that other cellular virulence pathways may be directly or indirectly addressed. This opens an intriguing perspective of a multifaceted virulence reduction effective also for MRSA strains.

Acknowledgements

This work was funded by the DFG (SI1096/8-1) and the Center for Integrated Protein Science Munich. K. M. L. thanks the Korea Research Institute of Chemical Technology. We thank Dorte Frees for providing 8325-4 wt and ΔclpX strains. We also thank Carola Seyffarth and Jessica Pryzgodda (FMP Berlin) for realization of the HTS, Katja Bäuml and Mona Wolff for excellent technical assistance, Melina Vollmer and Theresa Rauh for help with experiments and Markus Lakemeyer and Matthias Stahl for critical proofreading. The MS data have been deposited to the ProteomeXchange Consortium via the PRIDE[12] partner repository with the dataset identifier PXD007259.

Keywords: Antivirulence • high-throughput screening • proteolysis • proteomics • *Staphylococcus aureus*

[1] F. D. Lowy, *N Engl J Med* **1998**, *339*, 520-532.

[2] A. Zecconi, F. Scali, *Immunol Lett* **2013**, *150*, 12-22.

[3] a) A. E. Clatworthy, E. Pierson, D. T. Hung, *Nat Chem Biol* **2007**, *3*, 541-548; b) C. I. Liu, G. Y. Liu, Y. Song, F. Yin, M. E. Hensler, W. Y. Jeng, V. Nizet, A. H. Wang, E. Oldfield, *Science* **2008**, *319*, 1391-1394; c) D. A. Rasko, V. Sperandio, *Nat Rev Drug Discov* **2010**, *9*, 117-128.

[4] a) D. Frees, K. Sorensen, H. Ingmer, *Infect Immun* **2005**, *73*, 8100-8108; b) A. Michel, F. Agerer, C. R. Hauck, M. Herrmann, J. Ullrich, J. Hacker, K. Ohlsen, *J Bacteriol* **2006**, *188*, 5783-5796; c) J. Krysiak, M. Stahl, J. Vomacka, C. Fetzer, M. Lakemeyer, A. Fux, S. A. Sieber, *J Proteome Res* **2017**, *16*, 1180-1192.

[5] D. Frees, S. N. Qazi, P. J. Hill, H. Ingmer, *Mol Microbiol* **2003**, *48*, 1565-1578.

[6] a) R. T. Sauer, T. A. Baker, *Annu Rev Biochem* **2011**, *80*, 587-612; b) Z. Maglica, K. Kolygo, E. Weber-Ban, *Structure* **2009**, *17*, 508-516.

[7] a) L. Cheng, T. A. Naumann, A. R. Horswill, S. J. Hong, B. J. Venters, J. W. Tomsho, S. J. Benkovic, K. C. Keiler, *Protein Sci* **2007**, *16*, 1535-1542; b) S. M. McGillivray, D. N. Tran, N. S. Ramadoss, J. N. Alumasa, C. Y. Okumura, G. Sakoulas, M. M. Vaughn, D. X. Zhang, K. C. Keiler, V. Nizet, *Antimicrob Agents Chemother* **2012**, *56*, 1854-1861.

[8] a) T. Böttcher, S. A. Sieber, *J Am Chem Soc* **2008**, *130*, 14400-14401; b) F. Weinandy, K. Lorenz-Baath, V. S. Korotkov, T. Bottcher, S. Sethi, T. Chakraborty, S. A. Sieber, *ChemMedChem* **2014**, *9*, 710-713.

[9] A. Pahl, et al., *Angew Chem Int Ed Engl* **2015**, *54*, 15892-15896.

[10] Y. I. Kim, R. E. Burton, B. M. Burton, R. T. Sauer, T. A. Baker, *Mol Cell* **2000**, *5*, 639-648.

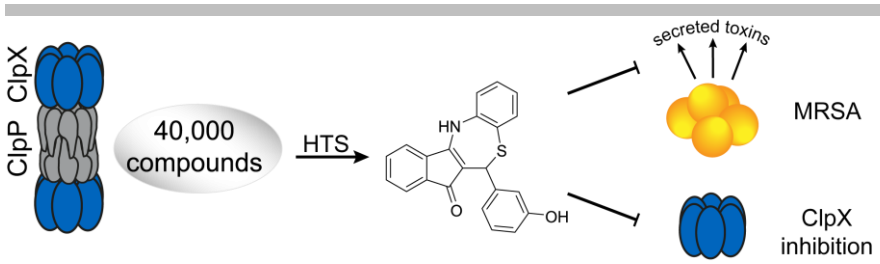
[11] P. P. Geurink, L. M. Prely, G. A. van der Marel, R. Bischoff, H. S. Overkleeft, *Top Curr Chem* **2012**, *324*, 85-113

[12] J. A. Vizcaíno, et al., *Nucleic Acids Res* **2016**, *44*, D447–D456.

10.1002/anie.201708454

WILEY-VCH

COMMUNICATION



A high-throughput screen against the ClpXP protease revealed the first non-covalent inhibitors of the chaperone ClpX. Binding of the inhibitors induces disruption of the ClpX hexamer and even of the whole ClpXP proteolytic complex. Treatment of *Staphylococcus aureus* with the compounds leads to a global down-regulation of a plethora of secreted virulence factors.

*Christian Fetzer, Vadim S. Korotkov, Robert Thänert, Kyu Myung Lee, Martin Neuenschwander, Jens Peter von Kries, Eva Medina, Stephan A. Sieber**

Page No. – Page No.

A Chemical Disruptor of the ClpX Chaperone Complex Attenuates Multiresistant *Staphylococcus aureus* Virulence