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Supplemental Information

Clemizole inhibits CrtN-Driven staphyloxanthin biosynthesis in *Staphylococcus aureus* to enhance host immune clearance

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31 **Supplementary Table 1. Strains used in this study**

Strains	Source
MRSA USA300 LAC	ATCC BAA-1717
ATCC 29213	ATCC 29213
ATCC33591	ATCC 33591
SA37	<i>S. aureus</i> clinical isolate
SA28	<i>S. aureus</i> clinical isolate
SA1B3G	<i>S. aureus</i> clinical isolate
Newman	This study
pET28a:: <i>crtN</i> -BL21 (DE ₃)	This study
pET28a:: <i>crtN</i> (51-55A)-BL21 (DE ₃)	This study
pET28a:: <i>crtN</i> (M221A)-BL21 (DE ₃)	This study
pET28a:: <i>crtN</i> (F431A)-BL21 (DE ₃)	This study
pET28a:: <i>crtN</i> (F443A)-BL21 (DE ₃)	This study
pET28a:: <i>crtM</i> -BL21 (DE ₃)	This study
<i>Pseudomonas aeruginosa</i> PAO1	ATCC 15692

32 ATCC, American Type Culture Collection; MRSA, methicillin-resistant *Staphylococcus aureus*
33 All clinical isolates were collected under institutional ethical approval, and no patient-identifiable
34 information was used in this study

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49 **Supplementary Table 2 Primers used in this study.**

Primers	Nucleotide sequence (5'→ 3')	Purpose
<i>crtN</i>	Forward: GCGGGATCCATGAAGATTGCAGTAATTGGTG Reverse: GCGGTCGACGACTACTCCCTTATACGCCC	PCR amplification
<i>crtN</i> (51-55A)	Forward: ATGCGGCTGCCGCAGCTGTCATGATGCCAGATGTTTATAAAGAT Reverse: AGCTGCGGCAGCCGCATCAAATGTAAAGCCGTCTTTCTTT	Site-directed mutagenesis
<i>crtN</i> (M221A)	Forward: TAAAGGCGGTGCGTATGGCATGGCTCAAGGGC Reverse: CATACGCACCGCCTTTAATAAAATGCACACCA	Site-directed mutagenesis
<i>crtN</i> (F431A)	Forward: CCAAATGATGCTGAGCAAACGTATCATGCGAAATT Reverse: TGCTCAGCATCATTTGGCGTAAAGATTGTTTCT	Site-directed mutagenesis
<i>crtN</i> (F443A)	Forward: TTCGGCAGCCGGTTTAATGCCAACTTTAGCGCA Reverse: ATTAAACCGGCTGCCGAACCAAATTTTCGCATGATAC	Site-directed mutagenesis
<i>crtM</i>	Forward: GCGGGATCCATGGCATTTCATATAGGAGG Reverse: GCGGTCGACACTTCGTCCAAGTATTTCAAT	PCR amplification
<i>crtN</i> -q	Forward: AATGCTGAACAAGAGTAATC Reverse: AGTGAATGGTGACATAAGA	Quantitative PCR
<i>16 sRNA</i> -q	Forward: ACTCCTACGGGAGGCAGCAG Reverse: ATTACCGCGGCTGCTGG	Quantitative PCR

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67 **Supplementary Table 3. Information of screened compounds.**

No.	Compound name	CAS	Molecular weight
1	IPI-145	1201438-56-3	416.86
2	Roscovitine	186692-46-6	354.45
3	PA-824	187235-37-6	359.26
4	GalanthamineHBr	1953-04-4	368.27
5	Ramelteon	196597-26-9	259.34
6	Fludarabine	21679-14-1	285.23
7	Didanosine	69655-05-6	236.23
8	EntecavirHydrate	209216-23-9	295.29
9	Vatalanib (PTK787) 2HCl	212141-51-0	419.73
10	LY2835219	1231930-82-7	602.7
11	MianserinHCl	21535-47-7	300.83
12	ImatinibMesylate	220127-57-1	589.71
13	Doxorubicin (Adriamycin) HCl	25316-40-9	579.98
14	Pelitinib	257933-82-7	467.92
15	Bumetanide	28395-03-1	364.42
16	Cediranib (AZD217)	288383-20-0	450.51
17	E64d	88321-09-9	342.43
18	Decitabine	2353-33-5	228.08
19	5-Azacytidine	320-67-2	244.2
20	Macitentan	441798-33-0	588.27
21	(-) Huperzine	102518-79-6	242.3
22	Carfilzomib	868540-17-4	719.91
23	MEK162	606143-89-9	441.23
24	Etomidate	33125-97-2	244.29
25	Asunaprevir	630420-16-5	748.29
26	AXL1717	477-47-4	414.41
27	Sulfasalazine	599-79-1	398.39

28	Cidofovir	113852-37-2	279.19
29	IbuprofenLysine	57469-77-9	352.47
30	Entacapone	130929-57-6	305.29
31	Boceprevir	394730-60-0	519.68
32	Capsaicin	404-86-4	305.41
33	Cariprazine	839712-12-8	427.41
34	Cetirizine	83881-51-0	388.89
35	CholineFenofibrate	856676-23-8	421.91
36	Cinacalcet	226256-56-0	357.41
37	Clemizole	1163-36-6	362.3
38	CO-1686 (AVL-301)	1374640-70-6	555.55
39	Cobimetinib	934660-93-2	531.31
40	Colchicine	64-86-8	399.44

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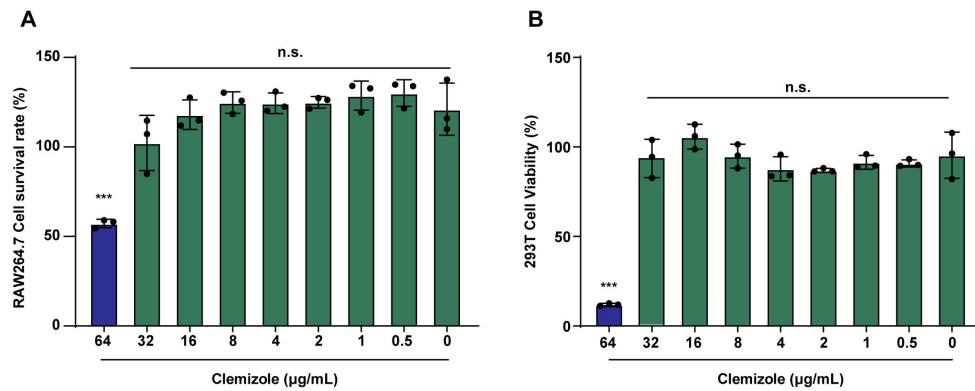
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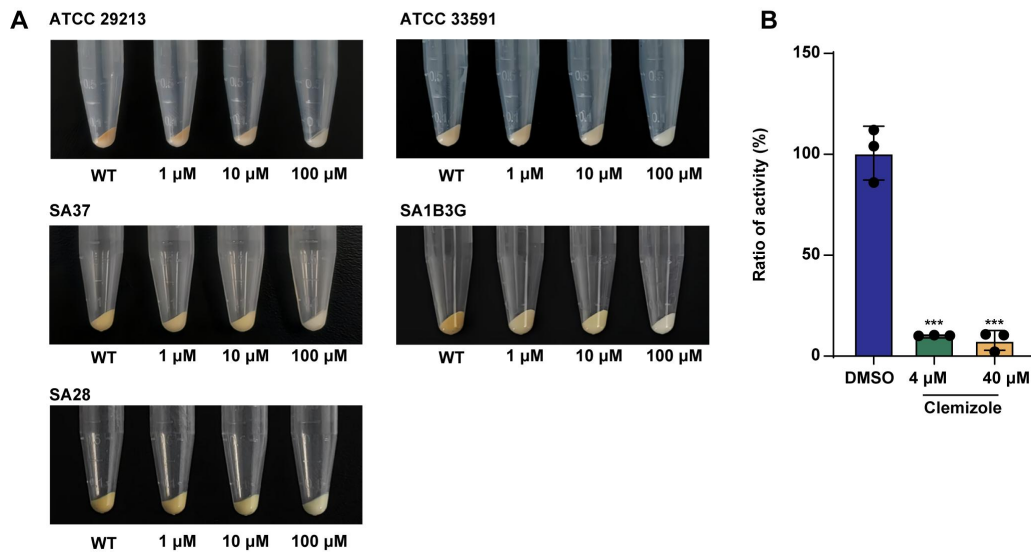
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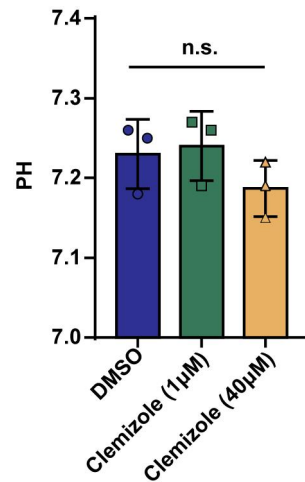
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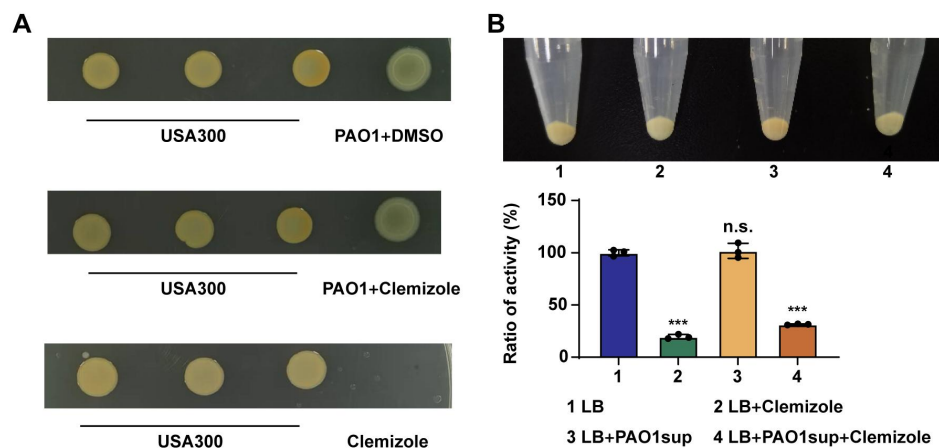
Supplementary Figure 1. Dose-response analysis of clemizole on cell viability. (A) RAW 264.7 macrophages and (B) 293T cells were treated with clemizole (0-64 µg/mL) for 24 hours. Cell viability was assessed using the CCK-8 assay. Data are presented as mean $\pm$ SD (n = 3). n.s., not significant. Unless otherwise specified, all experiments were performed with n = 3 biologically independent samples.



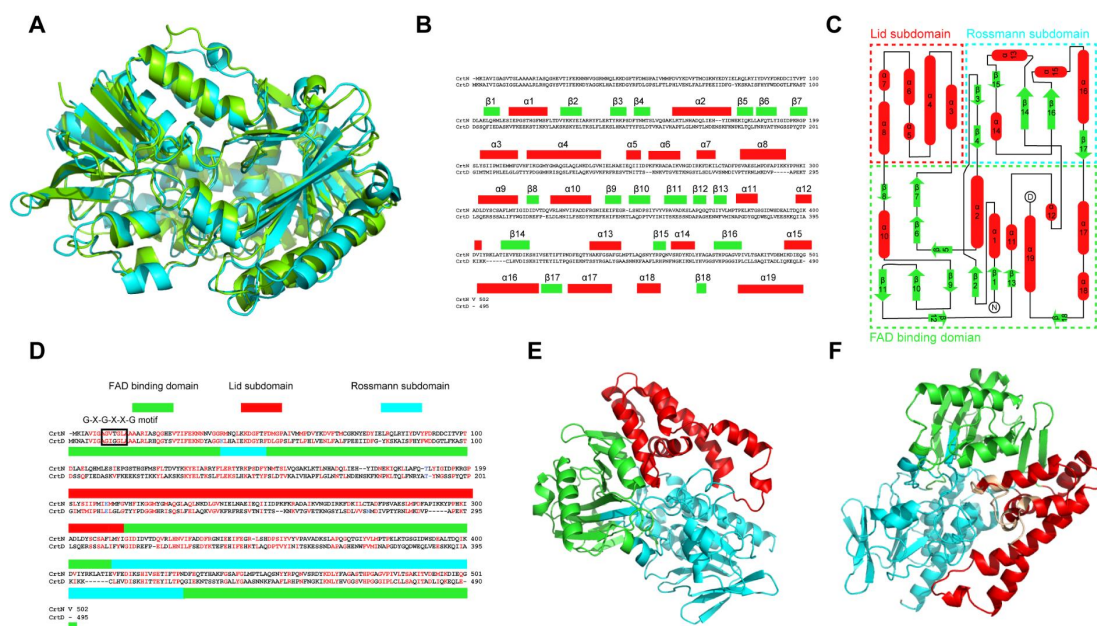
Supplementary Figure 2. Clemizole inhibits staphyloxanthin biosynthesis in clinical *S. aureus* isolates and directly targets CrtN enzyme activity. (A) Representative images of pigment pellets extracted from *S. aureus* reference strains (ATCC 29213, ATCC 33591) and clinical isolates (SA37, SA1B3G, SA28) after 16 h incubation with clemizole (1, 10, or 100 μ M) or DMSO. (B) Reversibility of clemizole-mediated CrtN inhibition. CrtN was either directly incubated with 4 μ M clemizole or pre-incubated with 40 μ M clemizole followed by dilution to a final concentration of 4 μ M prior to enzymatic assay. Enzyme activity is expressed relative to DMSO control (mean $\pm$ SD, n = 3). *** p < 0.001. Unless otherwise specified, all experiments were performed with n = 3 biologically independent samples.



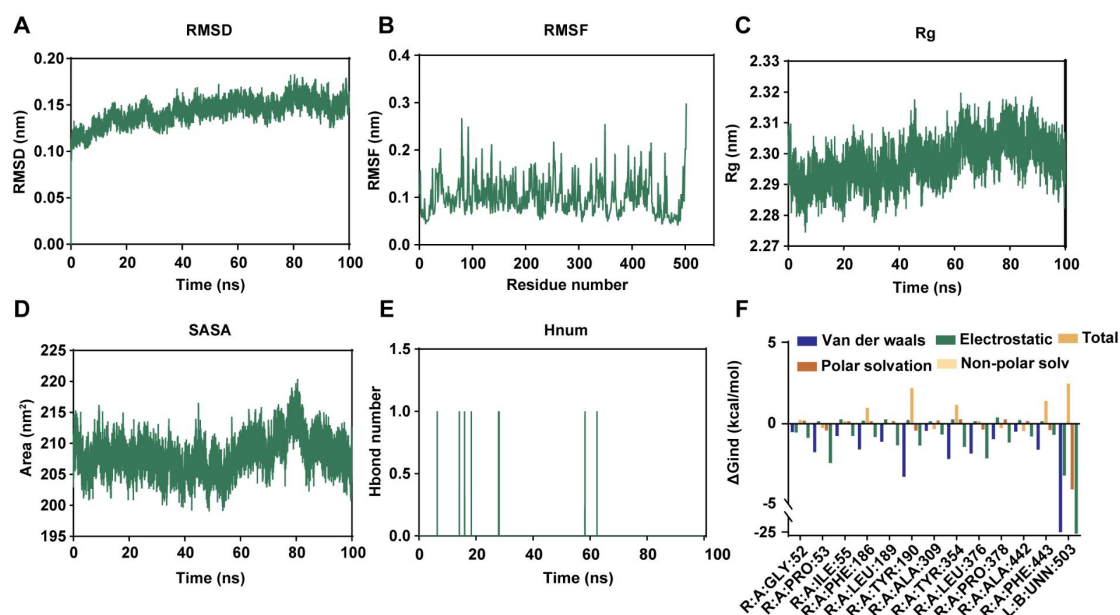
Supplementary Figure 3. Clemizole-HCl does not alter the culture medium pH at working concentrations. The pH of tryptic soy broth (TSB) containing DMSO or Clemizole-HCl (1 µM and 40 µM) was measured after incubation under standard conditions. No significant change in pH was observed among the groups, indicating that Clemizole-HCl does not affect medium acidity within the buffered system (n.s., not significant). Unless otherwise specified, all experiments were performed with n = 3 biologically independent samples.



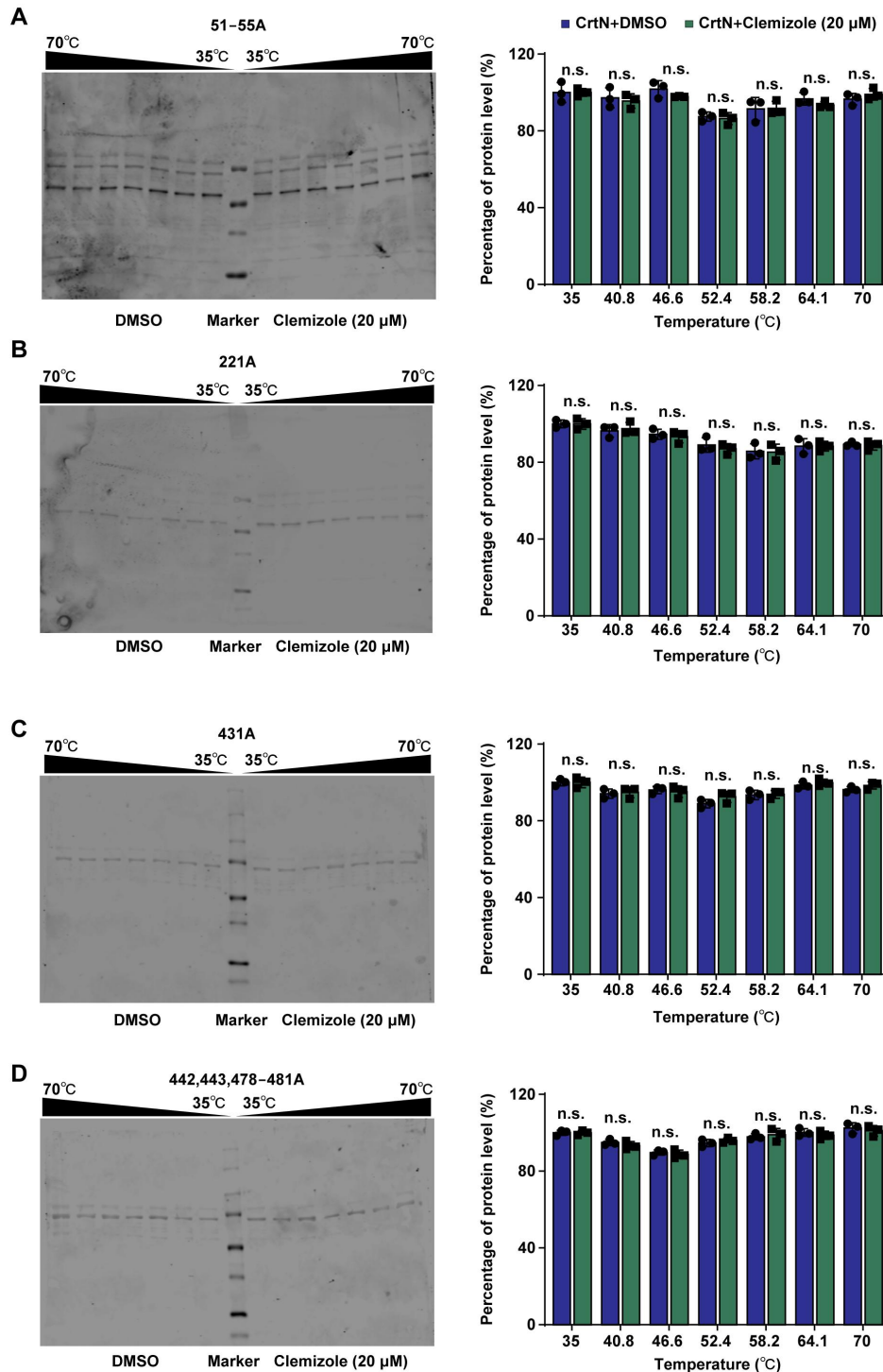
Supplementary Figure 4. Clemizole does not interfere with *P. aeruginosa*-mediated enhancement of *S. aureus* pigment production. (A) Large-colony proximity assay. *S. aureus* USA300 was plated adjacent to *P. aeruginosa* PAO1 on agar containing DMSO or clemizole (10 μ M). Clemizole had no effect on PAO1-induced enhancement of USA300 STX production. (B) Pigment extraction from USA300 cultured in (1) LB, (2) LB + clemizole, (3) LB + PAO1 culture supernatant, and (4) LB + PAO1 supernatant + clemizole. All experiments were performed in triplicate with consistent results. Unless otherwise specified, all experiments were performed with $n = 3$ biologically independent samples.



Supplementary Figure 5. Homology-based structural prediction of CrtN domain organization. (A) Structural superposition of *S. aureus* CrtN (green) and its homolog CrtD (blue), illustrating the conserved overall fold. (B) Sequence–structure alignment of CrtN and CrtD, with annotated α -helices and β -strands indicating the distribution of secondary structural elements. (C) Schematic representation of the CrtN secondary structure: α -helices (red cylinders) and β -strands (green arrows), with the FAD-binding domain, Rossmann-fold subdomain, and lid subdomain outlined by green, blue, and red dashed rectangles, respectively. (D) Domain mapping onto the linear secondary structure diagram of CrtN: FAD-binding domain (green), Rossmann fold (blue), and lid subdomain (red). (E) Ribbon representation of the CrtN homology model, colored by domain as in (D). (F) AlphaFold confidence heatmap projected onto the CrtN model; regions with low prediction confidence (pLDDT < 70) are highlighted in yellow, primarily localizing within the lid subdomain.

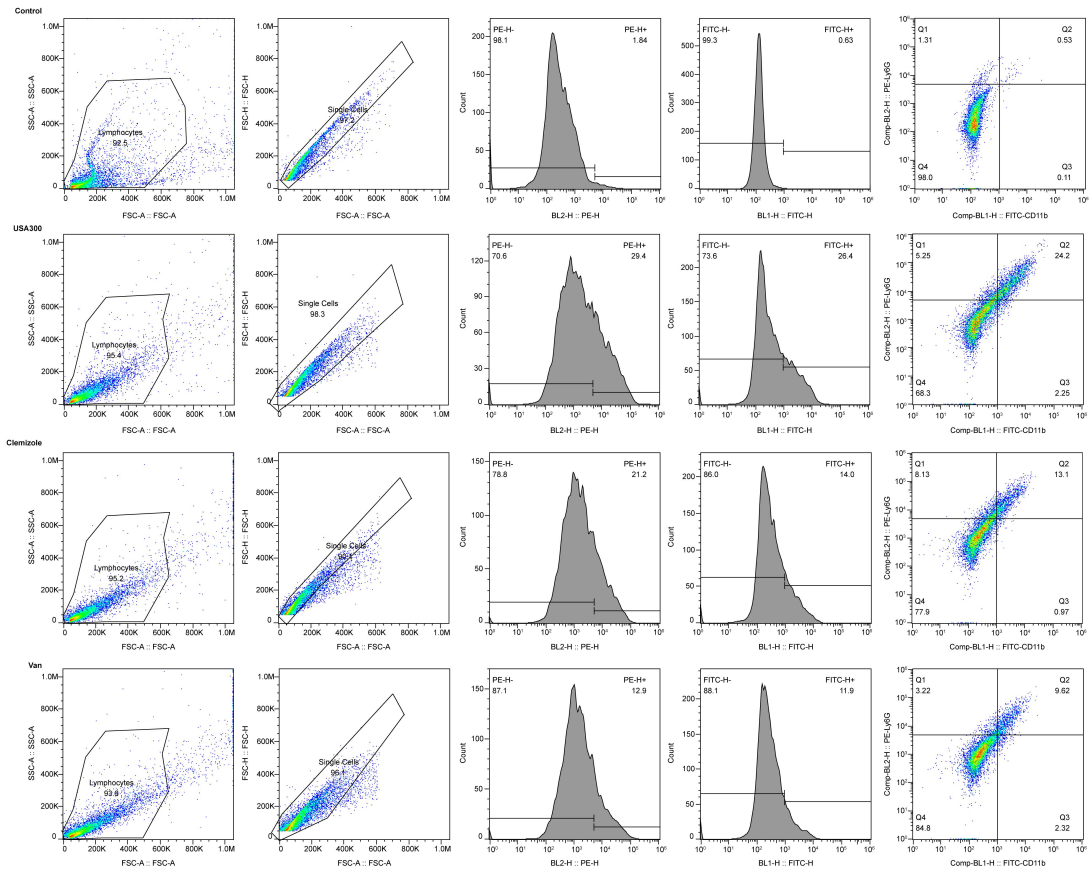


Supplementary Figure 6. Molecular dynamics simulation of the clemizole-CrtN complex over a 100-ns trajectory. (A-D) Time evolution of the backbone root-mean-square deviation (RMSD, Å), per-residue root-mean-square fluctuation (RMSF, Å), radius of gyration (Rg, Å) and solvent-accessible surface area (SASA, Å²) of the clemizole-CrtN complex, demonstrating structural stability and compactness throughout the simulation. (E) Number of hydrogen bonds formed between clemizole and CrtN over time. (F) Per-residue decomposition of the binding free energy (ΔG_{bind} , kcal · mol⁻¹) for the clemizole-CrtN complex, partitioned into van der Waals, electrostatic, polar solvation and nonpolar solvation contributions. All plots represent data from the equilibrated portion of the 100-ns trajectory (n = 1 simulation).



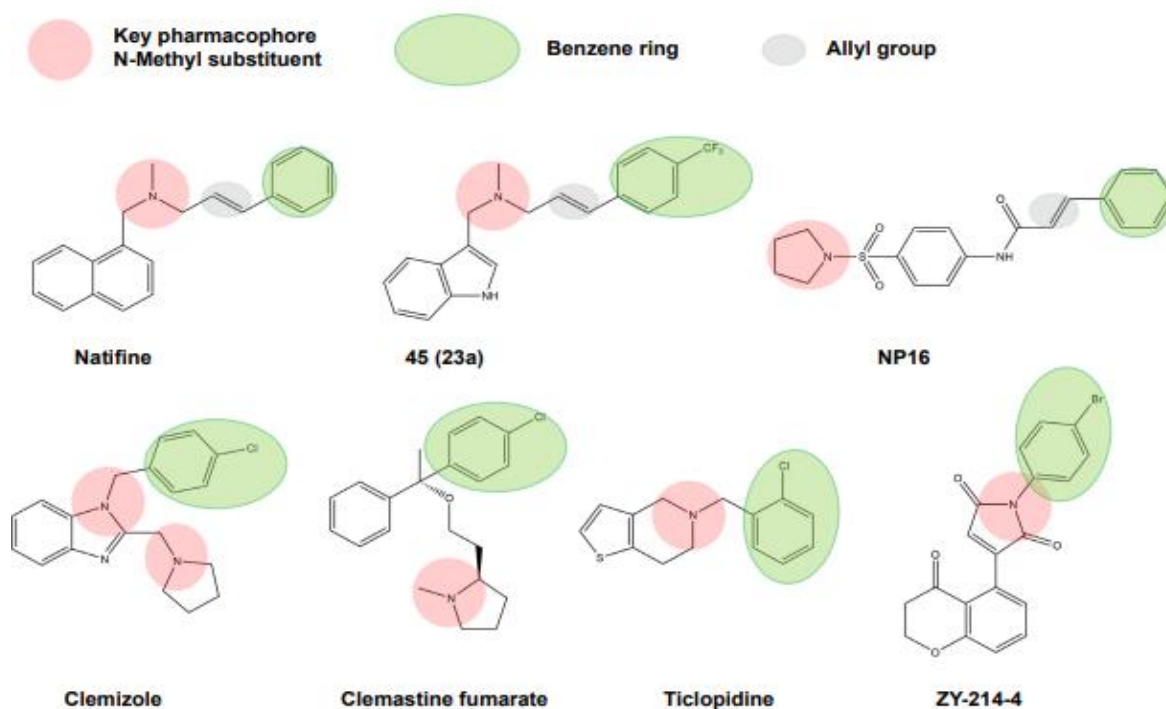
Supplementary Figure 7. Clemizole does not alter the thermal stability of CrtN mutants, as determined by TSA. Panels correspond, from top to bottom, to (A) CrtN-51-55A, (B) M221A, (C) F431A, and (D) F443A. Lysates containing each CrtN variant were preincubated with DMSO or 20 μM clemizole, followed by exposure to a temperature gradient (35-70 °C). After centrifugation to separate soluble protein, the supernatants were analyzed by SDS-PAGE and immunoblotting.

Densitometric quantification of soluble CrtN (right panels) is presented as the percentage remaining at each temperature relative to the 35 °C baseline (mean $\pm$ SD, n = 3). Two-way ANOVA revealed no significant differences (n.s.) between the DMSO and clemizole treatments across the full temperature range for any mutant. Unless otherwise specified, all experiments were performed with n = 3 biologically independent samples.



Supplementary Figure 8. Flow cytometry gating strategy for Ly6G⁺CD11b⁺ neutrophils in a murine burn injury model.

Single cell suspensions were prepared from mouse samples following burn injury. After exclusion of debris and doublets based on forward scatter and side scatter properties, live leukocytes were gated. Neutrophils were subsequently identified as Ly6G⁺CD11b⁺ cells. Representative flow cytometry plots illustrate the sequential gating strategy used for quantitative analysis of neutrophil populations in the burn injury model.



Supplementary Figure 9. Structure-activity relationship analysis of CrtN inhibitors.

Representative CrtN-targeting compounds exhibit common structural features associated with inhibitory activity. Key pharmacophores, including N-methyl substituents (highlighted in red), are consistently present across active compounds, indicating their essential role in CrtN binding. Benzene rings (green) are frequently observed, contributing to hydrophobic interactions and molecular stability. Additionally, allyl groups (gray) appear in select molecules and may enhance compound flexibility or membrane permeability. A comparison of diverse scaffolds, such as Natifine, Clemizole, and ZY-214-4, revealed a conserved pharmacophore pattern, supporting the rational design of potent CrtN inhibitors.