Supplementary Figures

Title: “Pyrazolopyrimidinones, a novel class of copper-dependent bactericidal antibiotics against multi-drug resistant S. aureus”

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Figure S1: Changes in sensitivity of ΔcopA and MNG3 to Cu. a) Alignment of the predicted copA promoter region of WT S. aureus strain Newman to that of the Cu resistant mutant, MNG3. The promoter was predicted using the PromoterHunter online software. The G to T mutation within the binding site of the copper-sensitive transcriptional repressor CsoR is shown in red. The last base pair shown is 25 bp upstream of the copA start site. b) A copA KO strain was tested for sensitivity to Cu. c) Expression of copA in WT S. aureus strain Newman (black) and MNG3 (gray) with and without 30 mins of Cu exposure. Data are expressed as fold change to WT in the absence of Cu. d) MNG3 was tested for sensitivity to Cu.
Figure S2: Calibration curve of pH responsive GFP. A series of phosphate-citrate buffers (pH 5.0 to 8.5) containing the lysate of S. aureus cells expressing the pH sensitive GFP were read. Error bars represent the coefficient of variation for the ratios.
Figure S3: Copper-dependency of 915’s anti-staphylococcal activity. Impact of BCS (500 μM) on 915’s inhibitory qualities against S. aureus Newman in the presence and absence of copper sulfate (50 μM).
Figure S4: Determination of metal binding by 915 using UV/VIS. a) UV/Vis spectrum of 915, 915+CuBr$_2$, 915+ZnSO$_4$, 915+FeCl$_2$, and CuBr$_2$ in 25μM HEPES buffer (pH=7.2). a) 1.54 x 10$^{-5}$ M CuBr$_2$, b) 8.58 x 10$^{-4}$ M PZP-915, c) 1.54 x 10$^{-5}$ M CuBr$_2$ + 2.14 x 10$^{-4}$ M PZP-915, d) 1.54 x 10$^{-5}$ M FeCl$_2$ + 2.14 x 10$^{-4}$ M PZP-915, e) 1.54 x 10$^{-5}$ M ZnSO$_4$ + 2.14 x 10$^{-4}$ M PZP-915. Typical absorption maxima were discerned at the following wavelength: PZP-915: 320 nm, PZP-915 + CuBr$_2$: 285nm and 315 nm, PZP-915 + FeCl$_2$: 282nm and 315nm (shoulder), PZP-915 + ZnSO$_4$: 282nm and 315nm (shoulder) b) Job plot of Fe(II), Cu(II), and Zn(II) with 915 at absorbance maximums. All three complexes have M(PZP915)$_3^{2+}$ stoichiometry.
Figure S5: Toxicity of Fe and Zn on *S. aureus*. Sensitivity of *S. aureus* to FeCl$_3$ and ZnCl$_2$. 

![Graph showing the toxicity of Fe and Zn on S. aureus.](image-url)
Figure S6: Killing by 915+Cu requires low micromolar amounts of copper. *S. aureus* Newman was treated with indicated concentrations of CuSO$_4$ and PZP 915 in standard assay medium. At the indicated times, 5 µl of the treated culture were removed and spotted for recovery onto MH-agar plates. Images were taken after overnight incubation at 37°C.
Figure S7: Longer treatment with 915 does not drastically increase membrane permeability. Membrane integrity was monitored using the membrane impermeable fluorescent indicator probe TO-PRO-3. Heat killed S. aureus cells served as positive control. A small number of live cells were added prior staining for control purposes. At the time when TO-PRO-3 fluorescence was taken, cells were exposed to Cu, 915 or 915+Cu for 8h. Treatments were 10 μM 915 + 50 μM Cu (blue), 10 μM 915 (green), 50 μM Cu (yellow), untreated (orange), and Heat-killed (HK, grey). Membrane integrity staining after a 30 min treatment is shown in Fig. 4B.
Figure S8: *S. aureus* metallomics response to 915 treatment. 

**a)** Fold-change of the metal content of *S. aureus* treated with 50 μM Cu (black), 10 μM 915 (blue), or 10 μM 915 + 50 μM Cu (pink) are shown. Fold change was calculated as a ratio of the amount of the indicated metal for the experimental value and untreated value. Dotted lines represent a +2 fold-change or -2 fold-change. Error bars represent the coefficient of variation. 

**b)** Percent difference of total cations measured in comparison to the control. 50 μM Cu (black), 10 μM 915 (light grey) 10 μM 915 + 50 μM Cu (dark grey). Error bars represent the variance. 

**c)** The amounts of each cation measured are stacked to represent the change in each metal after treatment. Cu (black), K (blue), Na (grey), Zn (dark pink), Fe (orange), Mn (purple), Ca (light pink), and Mg (green). Error bars represent standard deviation. Mn levels for the 915 and 915 + Cu treatments reached below the limit of detection, and so the bars represent the limit of detection.
Figure S9: Recovery of *S. aureus* copper-treated cells on MH-agar. *S. aureus* Newman was treated with indicated concentrations of CuSO$_4$ in standard assay medium. At the indicated times, 5 µl of the treated culture were removed and spotted for recovery onto MH-agar plates. Images were taken after overnight incubation at 37°C.
Figure S10: PZP 915 synthesis. Synthesis of 5-benzyl-3-(4-chlorophenyl)-2-methyl-4H,7H-pyrazolo[1,5-a]pyrimidin-7-one in three linear steps.
Figure S11: PZP 915 confirmation by $^1$H-NMR. $^1$H-NMR Spectrum of 5-benzyl-3-(4-chlorophenyl)-2-methylpyrazolo[1,5-a] pyrimidin-7(4H)-one (4).
Figure S12: PZP 915 confirmation by $^{13}$C-NMR. $^{13}$C-NMR Spectrum of 5-benzyl-3-(4-chlorophenyl)-2-methylpyrazolo[1,5-a]pyrimidin-7(4H)-one (4).