Supplementary Material

Synergetic antimicrobial activity and mechanism of clotrimazole linked CO-Releasing Molecules

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SUPPLEMENTARY TABLES
Table S1. FTIR data on CO stretching vibrations of the studied CORMs

| Compound          | ν (CO) (cm⁻¹) |
|-------------------|--------------|
| MnBpyCtz          | 2038, 1941   |
| ReBpyCtz          | 2029, 1936, 1914 |
| MnBiqCtz          | 2031, 1943   |
| MnPybzimCtz       | 2024, 1906   |
| MnBpydinonCtz     | 2031, 1929   |
| MnPyNHCCtz        | 2030, 1942, 1919 |
| MnBisNHCCtz       | 2013, 1927, 1899 |
Table S2. Percentage of growth of *E. coli, S. enterica* and *S. aureus* after exposure to CORMs

| Compound         | *E. coli* |          | *S. enterica* |          | *S. aureus* |          |
|------------------|-----------|----------|---------------|----------|-------------|----------|
|                  | 2 h       | 4 h      | 2 h           | 4 h      | 2 h         | 4 h      |
| MnBpyCtz         | 58.7±1.7  | 93.2±7.6 | 72.9±12.2     | 105.3±6.2| 7.5±0.9     | 2.2±0.6 |
| ReBpyCtz         | 45.3±8.6  | 27.4±10.6| 62.4±8.1      | 62.7±1.9 | 8.8±1.0     | 2.7±0.8 |
| MnBiqCtz         | 100.4±5.9 | 109.0±2.7| 106.9±11.0    | 114.1±32.2| 9.5±0.7     | 3.2±0.7 |
| MnPyBzimCtz      | 95.1±6.7  | 89.9±1.8 | 107.1±8.7     | 115.0±9.2| 28.7±4.2    | 24.6±4.4|
| MnBpydinonCtz    | 133.1±7.1 | 124.8±6.7| 148.0±18.0    | 149.1±38.2| 113.7±8.2   | 134.7±10.0|
| MnPyNHCCtz       | 54.5±6.6  | 49.5±17.7| 81.2±16.8     | 82.1±18.6| 22.5±3      | 3.0±0.7 |
| MnBisNHCCtz      | 62.7±9.2  | 53.9±8.7 | 67.9±3.8      | 78.0±3.7 | 23.5±3.9    | 3.5±0.8 |
| MnBpyBr          | 89 ± 6.0  | 119 ± 16 | 97.3 ± 1.3    | 110.2 ± 10.2| 98.5 ± 5.7   | 96.0 ± 10.4|
| ReBpyBr          | 91.5 ± 5.6| 103.4 ± 9.3| 94.1 ± 5.5    | 105.3 ± 11.3| 97.8 ± 10.4   | 92.0 ± 13.5|
| MnBisNHCBr       | 95.1±3.5  | 95.2±8.1 | 95.1±6.7      | 96.6±4.7 | 80.4±7.6    | 100.6±20.1|
| MnPyNHCl         | 96.5±6.0  | 88.0±10.2| 95.2±4.9      | 95.7±6.8 | 113.7±14.8  | 122.5±15.4|
| Ctz              | 136.9±17.2| 131.1±11.4| 129.7±14.4    | 110.6±3.7| 23.5±1.9    | 12.9±1.7 |

*E. coli* K12MG1655 (35 µM), *S. enterica* serovar Typhimurium SL1344 (35 µM) and *S. aureus* USA300 JE2 (MRSA) (10 µM) were exposed to CORMs, at the concentrations indicated in brackets, for 2 h and 4 h. Percentage of growth was determined in relation to the OD600 of untreated cells at the same growth stage. Error bars represent SD of, at least, three biological replicates.
**Figure S1. Non-conjugated CORMs do not release CO intracellularly.**

Fluorescence microscopy images of *S. aureus* MRSA cells exposed to the non-conjugated CORMs and incubated with the fluorescent probe COP-1. Representative images of fluorescent cells exposed to **ReBpyBr** (left) and **MnBpyBr** (right) and their corresponding bright field images. For each condition, images are representative of three biological samples.
Figure S2. Parallel bioreporter profiling of ReBpyCtz, Ctz and ReBpyBr.

Firefly luciferase bioreporter assay demonstrating liaI induction by ReBpyCtz. Luminescence values were recorded at time points predetermined by reference antibiotics (1 h for the PliaI and PypuA reporter, 1.5 h for PhelD, 3.5 h for PyorB and 4 h for PyheI) and normalised to the untreated control (100%, black, horizontal dashed line). At the MIC, the signal of the non-induced bioreporters falls below the background level due to impaired biosynthetic capacity of the treated cells. The indicated reference antibiotics known to induce the respective reporter signal were used as positive controls. Error bars represent the standard deviation (SD) of two biological replicates, each including two technical replicates. While ReBpyCtz clearly yielded a signal for the liaI bioreporter strain after 1 h of exposure, Ctz did not show induction in this setup. It is notable, that the bioreporter assay shown here is based on the firefly luciferase, which works by emission of a single luminescence flash upon external addition of the substrate luciferin at a given time point. For the liaI readout, luciferin was added after 1 h of exposure to the test compounds, yielding an intense but short burst of light. One hour of cell exposure is not sufficient for Ctz to yield a signal. Notably, in another bioreporter assay conducted in this study (Fig. 6E) using the bacterial luciferase operon of Photorhabdus luminescens, which encodes not only the luciferase itself but enzymes regenerating the substrate, continuous recording of luminescence was possible. In this alternative assay, Ctz also induced the liaI lux-bioreporter strain, although 2 h of cell exposure were required for induction and the signal strength was substantially lower than that noted for ReBpyCtz.
Figure S3. ReBpyCtz and Ctz trigger depolarization of the bacterial membrane in S. aureus NCTC 8325 at elevated concentrations.

Time-resolved effect of indicated concentrations of Ctz, ReBpyBr or ReBpyCtz on the membrane potential in S. aureus NCTC 8325 as determined by DiOC2(3) staining. Compound concentrations were chosen in relation to the respective MICs. CCCP (5 μM, 0.4x MIC) was used as a positive and DMSO (1%) as a negative control. The arrow indicates the time point of compound addition. Error bars represent the SD of four biological replicates.
Figure S4. The membrane integrity of *S. aureus* NCTC8325 is not severely compromised by any of the tested compounds.

Exposure to either Ctz (16 μg/ml, 4x MIC), **ReBpyCtz** (1 μg/ml, 4x MIC) or **ReBpyBr** (64 μg/ml) for 120 min followed by staining with the membrane permeant Syto9 (green) and the membrane impermeant propidium iodide (PI, red). Nisin (100 μg/ml, crude preparation) served as a positive control, and DMSO (1%) as a negative control. Images taken either in the brightfield (left column) or in the green or red fluorescence channel (middle columns). The right column depicts an overlay of all three images. Scale bar, 2 μm.
Figure S5. ReBpyCtz leads to an intracellular accumulation of the peptidoglycan precursor UDP-MurNAc-pentapeptide in *S. aureus* ATCC 29213 after 30 min of treatment.

Acetone extracts of cytosolic fractions were analysed by LC-MS and the result of this analyses is shown. Upper two rows (measured in negative ionization mode), treatment with Ctz (40 µg/ml, 10x MIC), ReBpyCtz (2.5 µg/ml, 10x MIC) and ReBpyBr (64 µg/ml). Lower two rows (positive ionization mode), concentration series of ReBpyCtz (i.e., 0.5, 2.5 and 5 µg/ml, corresponding to 2x, 10x, 20x MIC, respectively). Vancomycin (2 µg/ml, 8x MIC) served as positive and DMSO (1%) as negative control. Grey, base peak chromatograms (BPC); red, extracted ion chromatograms for UDP-MurNAc-pentapeptide (UDP-MurNAc-PP).
**Figure S6. ReBpyBr does not inhibit HADA incorporation.**

HADA labelling of *B. subtilis* 168 after 30 min of pre-treatment with ReBpyBr (64 µg/ml) compared to the negative control treated with DMSO (0.5%). The top row shows an overlay of the phase-contrast and fluorescence channel (bottom row). The micrographs depict representative images and were adjusted to the same microscopic settings to allow for qualitative comparison. Scale bar, 5 µm.
Figure S7. Probing the effect of ReBpyCtz on lipid I biosynthesis.

(A) Scheme of the MraY-catalyzed biosynthesis of lipid I by transferring MurNAc-P from UDP-MurNAc-PP to the lipid carrier C55P. The assay was conducted with purified components in vitro. After the reaction was done, the lipid precursors were extracted and analysed by TLC. (B-C) TLC plate (B) and quantification (C) of the lipid I reaction product. ReBpyCtz, Ctz or ReBpyBr did not affect the formation of lipid I. Lipid I synthesis was fully inhibited by the addition of the positive control vancomycin. All test compounds were applied in a ten-fold molar excess over C55P. Error bars represent SD of three replicates. Regarding the MraY reaction, it is notable that it is reversible, meaning that the enzyme forms lipid I but that it can also decompose lipid I into its two substrates UDP-MurNAc-PP and C55-P. The assay shows that the lipid I amount formed in the presence of ReBpyCtz surpasses the untreated control. This finding is in accordance with the notion that ReBpyCtz forms a complex with lipid I, thereby shifting the equilibrium to the product.

Figure S8. Concentration-dependent induction of the luxABCDE operon from P. luminescens expressed from the Plial promotor in B. subtilis168.

Titration of the test compounds showed a concentration-dependent induction of the Plial promotor in B. subtilis for Ctz (B) and ReBpyCtz (C). The signal generated by ReBpyCtz was strong, starting already at 0.5 x MIC, and at 1x MIC it even surpassed the positive control vancomycin (D). The induction by Ctz (B), was somewhat lower but also very prominent at 2x MIC. In contrast, ReBpyBr showed no effect (A).
$^1$H NMR SPECTRA OF THE Ctz CONJUGATES

$^1$H NMR spectrum of fac-[Mn(CO)$_3$(Bpy)Ctz][OTf] in DMSO-d6
$^1$H NMR spectrum of fac-[Mn(CO)$_3$(Bpy)Ctz][OTf] in DMSO-d$_6$ (expansion)
$^1$H NMR spectrum of $\textit{fac}$-$\text{[Re(CO)}_3\text{(Bpy)Ctz}[\text{OTf}]$ in DMSO-d$_6$
$^1$H NMR spectrum of fac-[Re(CO)$_3$(Bpy)Ctz][OTf] in DMSO-d6 (expansion)
$^1$H NMR spectrum of fac-[Mn(CO)$_3$(Biq)Ctz][OTf]: in Acetone-d$_6$ (expansion)
$^1$H NMR spectrum of $\text{fac-}[\text{Mn(CO)}_3(2\text{-pyridyl-benzimidazole})(\text{Ctz})][\text{OTf}]$ in Acetone-d6 (expansion)
$^1$H NMR spectrum of $\text{fac-}[\text{Mn(CO)}_3(4,4'$-dinonyl-2,2'-bipyridyl)(Ctz)][\text{OTf}]$ in $\text{CH}_2\text{Cl}_2$-$d_2$
$^1$H NMR spectrum of $fac$-[Mn(N-methyl-N'-2-pyridylimidazol-2-ylidene)(CO)$_3$Ctz][OTf]: in DMSO-d$_6$
$^1$H NMR spectrum of $\text{fac-}[\text{Mn}(\text{N-methyl-N'\text{-2-pyridylimidazol-2-ylidene})(\text{CO})_3\text{Ctz}][\text{OTf}]]$: in DMSO-d$_6$ (expansion)
$^1$H NMR spectrum of $\text{fac-}[\text{Mn(methylene bis-}N\text{-methylimidazole-2-ylidene)}(\text{CO})_3(\text{Ctz})][\text{OTf}]$ in DMSO-d6

Note: The peak marked X corresponds to CH$_2$Cl$_2$ of crystallization which varies between samples.
$^1$H NMR spectrum of fac-[Mn(methylene bis-N-methylimidazole-2-ylidene)(CO)$_3$(Ctz)][OTf] in DMSO-d6
(expansion)
