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DCAP: A Broad-Spectrum Antibiotic That Targets the Cytoplasmic Membrane of Bacteria

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Supporting Information

ABSTRACT: Persistent infections are frequently caused by dormant and biofilm-associated bacteria, which often display characteristically slow growth. Antibiotics that require rapid cell growth may be ineffective against these organisms and thus fail to prevent reoccurring infections. In contrast to growth-based antimicrobial agents, membrane-targeting drugs effectively kill slow-growing bacteria. Herein we introduce 2-((3-(3,6-dichloro-9*H*-carbazol-9-yl)-2-hydroxypropyl)amino)-2-(hydroxymethyl)propane-1,3-diol (DCAP), a potent broad-spectrum antibiotic that reduces the transmembrane potential of Gram-positive and Gram-negative bacteria and causes mislocalization of essential membrane-associated proteins, including MinD and FtsA. Importantly, DCAP kills nutrient-deprived microbes and sterilizes bacterial biofilms. DCAP is lethal against bacterial cells, has no effect on red blood cell membranes, and only decreases the viability of mammalian cells after ≥6 h. We conclude that membrane-active compounds are a promising solution for treating persistent infections. DCAP expands the limited number of compounds in this class of therapeutic small molecules and provides new opportunities for the development of potent broad-spectrum antimicrobial agents.

While the prevalence of multi-drug-resistant pathogens continues to rise, the rate at which new clinical antimicrobials are introduced has declined significantly.[1] To add to this dismal picture of combating infectious diseases, the treatment of persistent infections has been complicated by the phenotypes of pathogens.[2] Bacteria that grow very slowly are often associated with prolonged infections, and they are particularly tolerant of many of the clinically important classes of antibiotics that inhibit rapidly growing cells. For example, the *β*-lactam family of antibiotics inhibits enzymes involved in the synthesis of peptidoglycan and is thus most effective at targeting microbes that grow rapidly and continuously synthesize new cell wall.[3] Relying on antibiotics that require fast metabolism creates long-term problems, as dormant bacteria may survive antibiotic treatments, become predisposed to develop drug resistance, and cause a relapse.[1]

An effective strategy for combating slow-growing bacteria is to target the lipid membrane.[2] Proteomic analyses have shown that roughly one-third of all proteins in bacteria are associated with membranes.[4] Peripheral and integral membrane proteins participate in various essential cellular processes, including nutrient and waste transport, respiration, adhesion, mobility, cell−cell communication, and the transfer of genetic material.[2,4] Compounds that perturb these processes disrupt growth and the maintenance of cell homeostasis and may serve as potent therapeutic antimicrobial agents.[2,5]

Synthetic and naturally occurring small molecules that disrupt the bacterial membrane have been developed to treat persistent infections of mycobacterial and staphylococcal species.[2,6] This class of compounds exhibits multiple mechanisms of action, including inhibiting specific enzymatic processes in the membrane, decreasing the transmembrane potential ($\Delta\Psi$), and increasing membrane permeability. The increase in permeability perturbs the bacterial physiology and simultaneously facilitates the penetration of free radicals secreted by macrophages of the host immune system.[2]

The therapeutic benefit of membrane-active drugs has been demonstrated against dormant bacteria; however, there are no clear design rules for small molecules that are specific for bacterial versus eukaryotic membranes.[2] Many antibiotics in this class are ineffective against Gram-negative bacteria, presumably because of the outer membrane.[6] The identification of new broad-spectrum antibiotics that target bacterial membranes and the study of their mechanism of toxicity will provide an important step forward for this field.

Herein we describe the discovery and characterization of a new compound, 2-((3-(3,6-dichloro-9*H*-carbazol-9-yl)-2-hydroxypropyl)amino)-2-(hydroxymethyl)propane-1,3-diol (DCAP), that specifically targets the membranes of both Gram-positive and Gram-negative bacteria (Figure 1). We identified DCAP via a high-throughput inhibitor screen of the in vitro activity of MipZ, an ATPase that regulates division site placement in *Caulobacter crescentus*.[7] Using a strain of *C. crescentus* in which MipZ was translationally fused to yellow fluorescent protein (YFP), we found that treating cells with DCAP (20 μM) caused MipZ−YFP to mislocalize (Figure S3). At high concentrations of DCAP (≥75 μM), we observed cell lysis within minutes after treatment (Figure S4). This

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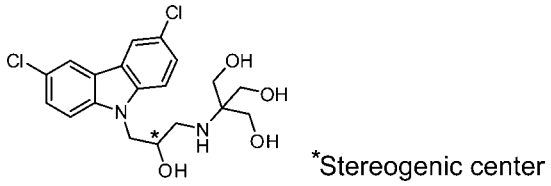

Figure 1. Chemical structure of DCAP.

observation suggested that DCAP may not specifically inhibit MipZ in the cell but instead alter the properties of the cell envelope.

To test this hypothesis, we measured $\Delta\Psi$ of two model bacteria, *C. crescentus* (Gram-negative) and *Bacillus subtilis* (Gram-positive), in the absence and presence of DCAP. As a positive control, we used carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). Hydrophobic weak acids such as CCCP transport protons and other cations across the membrane and decrease $\Delta\Psi$.[8] CCCP dissolves into the lipid bilayer, and the acidic form associates with a cation near a leaflet of the membrane. The neutral complex moves to the other leaflet and dissociates to release the cation. After dissociation of the complex, the ionophore becomes available to bind to another cation and transport it across the membrane.[9] This increased permeability to ions dissipates $\Delta\Psi$. To visualize changes in the membrane potential, we used the fluorescent probe 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$). $DiOC_2$ emits green fluorescence (λ = 530 nm) in the monomeric form, and its fluorescence emission maximum is red-shifted to λ = 576 nm upon self-association.[10] Molecules of $DiOC_2$ located inside cells reside in either the membrane or the cytoplasm. In the presence of a large $\Delta\Psi$, the number of positively charged molecules of $DiOC_2$ partitioned into the cytoplasm is greater than the number of molecules at the membrane.[11] The high local concentration in the cytoplasm causes $DiOC_2$ to aggregate and increase the fluorescence intensity ratio I_{576}/I_{530}. Conversely, I_{576}/I_{530} decreases when $\Delta\Psi$ is dissipated in bacteria.[10] A significant decrease in I_{576}/I_{530} was apparent after 20 min of treatment with CCCP and DCAP ($p < 0.001$) (Figure 2A,B), indicating that $\Delta\Psi$ dissipated rapidly. Antibiotics that do not target the bacterial membrane can decrease the potential over a long period of exposure (e.g., 3−4 h);[12] however, the rapid action of DCAP suggests that the dissipation of $\Delta\Psi$ is due to its direct effect on the inner membrane.

Next we explored the mechanism of action of DCAP. One possibility is that it functions as an ionophore similar to CCCP. Alternatively, it may increase the general permeability of the membrane. To investigate the mechanism, we used propidium iodide (PI) to label the DNA of cells with compromised membranes.[10] Ethanol-treated cells were intensely labeled with PI, while the DMSO control sample was not (Figure 2C,D). Treatment of cells with CCCP did not increase DNA labeling with PI; the fluorescence emission of these cells was similar to that of the DMSO sample. Addition of DCAP increased the fluorescence of cells labeled with PI, although the intensity was significantly lower than for ethanol-treated cells ($p < 0.001$). These results suggest that DCAP has at least two mechanisms of antimicrobial action: it decreases $\Delta\Psi$ by facilitating ion transport across the membrane and has a minor effect on the general permeability of the lipid bilayer. The bioactivity of DCAP may arise from its direct association with bacterial lipids or proteins in the membrane.

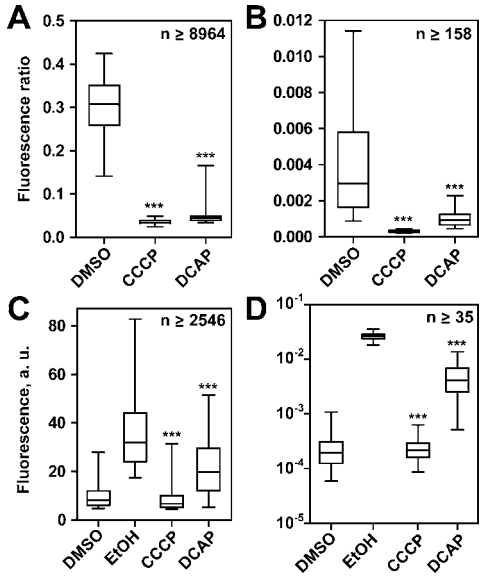

Figure 2. (A, B) Measurement of $\Delta\Psi$ using $DiOC_2$ in (A) *B. subtilis* and (B) *C. crescentus*. We calculated *p* values by comparing the data against the DMSO sample. (C, D) Measurement of membrane permeability using PI for (C) *B. subtilis* and (D) *C. crescentus*. We calculated *p* values by comparing the data against the EtOH sample. In the box plots, the top whisker represents 95%, the bottom whisker 5%, the top of the box 75%, and the bottom of the box 25%; the line inside the box indicates the median of each sample population. Three asterisks (***) refers to $p < 0.001$.

$\Delta\Psi$ was identified recently as an important parameter for the in vivo localization of division proteins associated with the bacterial membrane, including MinD and FtsA.[13] We found that the treatment of *B. subtilis* cells with either CCCP or DCAP altered the localization of a fusion of green fluorescent protein to MinD (GFP−MinD) compared with DMSO-treated cells (Figure 3A). MinD localizes at the poles of *B. subtilis* cells and guides division plane formation at the midcell. As division progresses, MinD accumulates at the midcell and marks the sites of future cell poles.[13] Treating *B. subtilis* cells expressing GFP−MinD with CCCP increased the diffuse fluorescence throughout the cell but had little effect on the location of the signal relative to the DMSO control (Figure S5A). In DCAP-

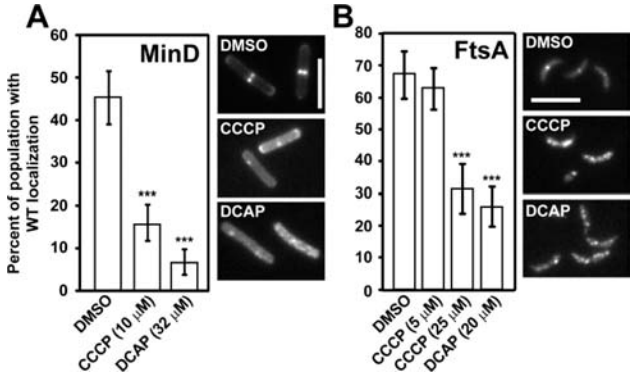

Figure 3. Analysis of membrane protein localization in bacteria (*** indicates $p < 0.0001$ relative to DMSO). Error bars represent the 95% confidence interval of the reported percentages. Representative images for each sample are shown (scale bars, 5 μm). (A) GFP−MinD in *B. subtilis* ($n \geq 252$ cells). (B) Venus-FtsA in *C. crescentus* ($n \geq 150$ cells). The middle image shows cells from the treatment with 25 μM CCCP.

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treated cells, GFP−MinD mislocalized: the number of fluorescent foci increased in some cells, while in others the fluorescence signal became more diffuse and was no longer concentrated at the poles (Figure S5A).

In addition to perturbing the localization of MinD in *B. subtilis*, CCCP and DCAP influenced the distribution of FtsA in *C. crescentus*. FtsA is a peripheral membrane protein that interacts with FtsZ and activates the recruitment of downstream division proteins.[14] FtsA resides at the pole opposite the stalk in nondividing *C. crescentus* cells and is recruited to the midcell as division begins.[14] To study cells at this stage of division, we synchronized cells expressing a fluorescent fusion of the protein Venus-FtsA and treated them with CCCP and DCAP. Treatment of *C. crescentus* cells with CCCP at its minimum inhibitory concentration (MIC) of 5 μM did not alter the localization of FtsA; however, a higher concentration of CCCP (25 μM) had a significant effect on the position of FtsA. Most cells treated with DCAP exhibited multiple peaks of FtsA fluorescence ($\geq$2) instead of the single peak (at either the pole or the midcell) observed in untreated cells (Figure S5B). The observation that DCAP causes mislocalization of membrane proteins in *B. subtilis* and *C. crescentus* is consistent with the hypothesis that DCAP decreases $\Delta\Psi$. Its effect is similar to that of CCCP, but it causes more severe protein mislocalization at its MIC, which may arise from its influence on membrane permeability.

After confirming the membrane-targeting activity of DCAP, we tested its efficacy against other bacteria. Table S2 shows that DCAP inhibits the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, and other clinical pathogens. Deleting one or more efflux pumps in *E. coli* and *P. aeruginosa* strains increased the sensitivity of cells to DCAP. Efflux pumps are active against a broad range of compounds and typically have three components: two transmembrane proteins (one each in the inner and outer membranes) and a periplasmic protein that connects them.[15] Deleting *tolC* in *E. coli* strain BW25113, which eliminates the outer-membrane component of an efflux pump, reduced the MIC of DCAP 4-fold, suggesting that the activity of DCAP in Gram-negative bacteria is largely due to its effect on the inner membrane.

In addition to its activity against actively growing bacteria, DCAP kills stationary-phase cells (Table 1). We tested this property of DCAP against *C. crescentus* and *Staphylococcus aureus*. We used *S. aureus* as a model Gram-positive bacterium rather than *B. subtilis* because *B. subtilis* can sporulate under starvation conditions and does not form robust biofilms on the plastic surfaces we used as substrates. To ensure that the bacteria were deprived of nutrients, we grew the cells to late stationary phase and suspended them in isotonic solutions lacking amino acids or sugars. After treating the cells with small molecules, we measured the cell viability over time by plating culture aliquots on nonselective, solid growth media. To test whether membrane-targeting drugs have a greater efficiency in killing cells that grow slowly, we compared the minimum concentration of antibiotic required to eliminate colony formation completely [the minimum stationary-bactericidal concentration (MSC)[16]] of CCCP and DCAP with that of ampicillin (Amp), which is lethal only to cells that are actively growing. We found that the MSC and MIC of DCAP were similar for each organism, while the efficacy of Amp was significantly reduced for stationary cells (Table 1). The MSC of Amp for *S. aureus* was 1000-fold higher than its MIC, while the MSC of Amp for *C. crescentus* was beyond the range of our measurements. CCCP inhibited the proliferation of *C. crescentus* cells regardless of their physiological status. However, the MSC of CCCP for *S. aureus* was >300-fold higher than the MIC. This dramatic decrease in the effectiveness of CCCP in *S. aureus* may be due to changes in membrane composition as the cells adjust their metabolism in nutrient-deprived conditions.[17] Overall, membrane-active CCCP and DCAP were more effective in killing stationary cells than Amp, whose mechanism of action is more specific toward cells with robust growth.

Table 1. Antibacterial Activities of DCAP, CCCP, and Amp

	C. crescentus			*S. aureus*		
	DCAP[a]	CCCP[a]	Amp[b]	DCAP[a]	CCCP[a]	Amp[b]
MIC	15	5	50	50	1.25	0.125
MSC	20	2.5	>400	75	>400	100
bMIC	20	5	100	100	2.5	0.8
MBEC	40	5	>400	>200	80	>200

[a]Concentration unit: μM. [b]Concentration unit: μg/mL.

We found that membrane-active compounds are also efficient at eradicating biofilm-associated cells (Table 1). Biofilms are implicated in a wide range of human diseases, including cystic fibrosis and urinary tract infections, and are particularly recalcitrant to antibiotics.[18] The heterogeneity in the physiology of cells in biofilms makes it possible for the bacterial communities to persist under stressful conditions.[19] To determine the efficacy of antibiotics against biofilms, we adopted protocols for measuring the minimum biofilm inhibitory concentration (bMIC) and the minimum biofilm eradication concentration (MBEC).[20] First, we formed biofilms on the surface of 96 individual plastic pins that protruded into the 96 wells of a microplate. The biofilms were exposed to compounds for 24 h, and we determined the bMIC for planktonic cell growth in the wells. Since the bMIC is a measurement of the rapid growth of freely suspended cells released from biofilms in the presence of antibiotics, the bMIC and MIC values did not differ significantly (Table 1).

After performing bMIC experiments, we transferred biofilms growing on the pins of the lid to nutrient media devoid of antibiotics to measure the MBEC for planktonic growth from biofilms in antibiotic-free nutrient media, which indicates whether the exposure to the antimicrobial agent used during the bMIC experiment sterilized biofilm-associated cells. The MBEC values were generally larger than the MICs and indicated an increased tolerance of stress exhibited by cells associated with biofilms. For *C. crescentus*, the MBEC values recapitulated the observed trend in the MSCs: CCCP and DCAP effectively eradicated biofilm cells, while Amp was not cytotoxic at the highest concentration tested (400 μM). CCCP was the only effective antibiotic against *S. aureus* biofilms. Since CCCP was not as effective as DCAP at killing stationary *S. aureus* cells, we suspect that this variability in efficacy of membrane-active drugs is caused in part by changes in membrane composition (i.e., membrane proteins and lipid content) at different developmental stages of bacterial cells.[17] Despite the variations in efficacy, we conclude that the comparison of MIC, bMIC, MSC, and MBEC measurements for the three antibiotics support the hypothesis that membrane-active drugs eradicate slow-growing bacteria more effectively than antibiotics that rely on growth-dependent mechanisms.

To test the toxicity of DCAP against mammalian membranes, we measured the hemolysis of rabbit red blood

cells (RBCs). We performed these experiments using conditions that closely mimicked the MIC and MSC assays. We treated RBCs with CCCP and DCAP at their MICs for 17 h, the time period used to determine the MICs of *C. crescentus* and *S. aureus*. After incubation, we measured the absorbance of heme released from lysed RBCs. We found that the MICs of CCCP and DCAP did not significantly disrupt RBC membranes (Figure 4A), although higher concentrations of DCAP (i.e., 50 μM) were moderately toxic to RBCs.

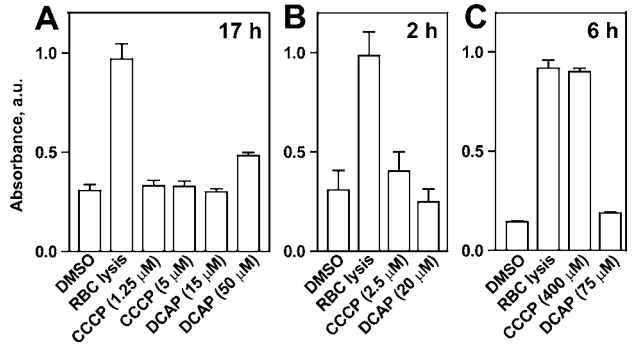

Figure 4. Rabbit RBC hemolysis assays using (A) the MIC conditions for *C. crescentus* and *S. aureus*, (B) the MSC for *C. crescentus*, and (C) the MSC for *S. aureus*. Columns represent means and error bars standard deviations for three replicates.

We also reproduced the MSC assay conditions to measure the toxicity against RBCs. First we determined the minimum time required to obtain the MSC for DCAP treatment of *C. crescentus* (2 h) and *S. aureus* (6 h) (data not shown). Using these times, we performed the hemolysis assays and observed no significant toxicity of DCAP against RBCs (Figure 4B,C). In contrast, CCCP was toxic to RBCs at high concentration (Figure 4C). These measurements indicate that DCAP does not appreciably perturb RBC membranes under conditions that are lethal to *C. crescentus* and *S. aureus*.

In addition to measuring the toxicity of membrane-active drugs on RBCs, we tested whether DCAP and CCCP dissipate $\Delta\Psi$ of mitochondria in mammalian cells. We used human epithelial kidney (HEK) cells as a model tissue culture cell line. As in the bacterial assays, we used $DiOC_2$ to probe the changes in $\Delta\Psi$ in the presence of the compounds. DCAP and CCCP slowly depolarized the mitochondrial $\Delta\Psi$ in HEK cells (Figure S6), but the efficiency of $\Delta\Psi$ dissipation was lower in mitochondria than in bacterial cells: the bacterial $\Delta\Psi$ was reduced ~10-fold within 20 min (when comparing median values in Figure 1A,B), but the mitochondrial $\Delta\Psi$ was reduced only 2-fold (the first data point in Figure S6).

This eventual decrease of mitochondrial $\Delta\Psi$ in HEK cells prompted us to investigate the viability of these cells upon treatment with DCAP and CCCP. The viability of HEK cells was minimally perturbed at short time intervals following treatment (2 h; Figure S7), while longer incubations decreased the cell viability. As CCCP and DCAP were similar in their ability to dissipate the mitochondrial $\Delta\Psi$ in these cells, we speculate that the toxicity of DCAP toward HEK cells may not be caused by its effect on the mitochondrial $\Delta\Psi$. We plan to investigate the basis of this toxicity of DCAP in future studies.

In summary, we have reported the discovery and characterization of DCAP, a membrane-active antimicrobial agent that kills bacteria by depolarizing $\Delta\Psi$ and increasing membrane permeability. These activities disrupt the organization and integrity of the bacterial membrane and mislocalize essential membrane-associated proteins. DCAP is inert to RBC membranes at concentrations at which it is a potent antibacterial agent. However, DCAP slowly reduces the mitochondrial $\Delta\Psi$ and becomes toxic to HEK cells. We plan to address the cytotoxicity of DCAP on mammalian cells by synthesizing and testing analogues. Studies with DCAP analogues may also provide insight into changes in the properties of membranes during the life cycle of bacteria and enable alterations in cell physiology to be correlated with the vulnerability of cells to membrane-active drugs. Finally, studies of the structure–function relationship of DCAP and other broad-spectrum compounds may provide design rules for potent membrane-targeting drugs that kill bacterial cells specifically.

■ ASSOCIATED CONTENT

Supporting Information

Methods and materials, DCAP characterization, microscopy images, and complete refs 3, 5, and 14. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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