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ARTICLE

Conjugating Berberine to a Multidrug Resistance Pump Inhibitor Creates an Effective Antimicrobial

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ABSTRACT In bacteria, multidrug-resistance pumps (MDRs) confer resistance to chemically unrelated amphipathic toxins. A major challenge in developing efficacious antibiotics is identifying antimicrobial compounds that are not rapidly pumped out of bacterial cells. The plant antimicrobial berberine, the active component of the medicinal plants echinacea and golden seal, is a cation that is readily extruded by bacterial MDRs, thereby rendering it relatively ineffective as a therapeutic agent. However, inhibition of MDR efflux causes a substantial increase in berberine antimicrobial activity, suggesting that berberine and potentially many other compounds could be more efficacious if an effective MDR pump inhibitor could be identified. Here we show that covalently linking berberine to INF_{55}, an inhibitor of Major Facilitator MDRs, results in a highly effective antimicrobial that readily accumulates in bacteria. The hybrid molecule showed good efficacy in a *Caenorhabditis elegans* model of enterococcal infection, curing worms of the pathogen.

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Efflux by multidrug-resistance pumps (MDRs) is a universal mechanism by which microorganisms resist a broad variety of antimicrobials (*1*–*4*). Bacterial MDRs are found in all microorganisms and make up five distinct independently evolved protein families (*5*). The first MDR pump described was the human ABC (ATP binding cassette)-family P-glycoprotein transporter (*6*), which protects a number of tissues from xenobiotics and is an essential component of the blood–brain penetration barrier (*7*–*9*). Overexpression of P-glycoprotein plays an important role in tumor resistance to chemotherapeutic agents (*10*). Although a few bacterial P-glycoprotein homologues have been described, such as the LmrA MDR of *Lactococcus lactis* (*11*), bacterial ATP-dependent MDRs are uncommon, and efflux of clinically relevant compounds is due primarily to the drug/proton antiporters of the resistance nodulation cell division (RND) type MDRs of Gram-negative species (*12*) and major facilitator (MF) MDRs present in all groups of microorganisms (*13*). Some of the MF MDRs, such as the *Staphylococcus aureus* QacA pump, are carried on transmissible genetic elements (*14*).

Unlike specialized transporters, MDRs recognize their substrates largely on the basis of polarity. In order to cross the lipid bilayer of the membrane, drugs must be amphipathic, containing both hydrophilic and hydrophobic components, whereas cytoplasmic compounds are hydrophilic, which prevents their escape from the cell (*15*, *16*). Any amphipathic compound could potentially be a toxin, providing a simple basis for an MDR pump to discriminate self from harmful foreign mol-

ecules. The crystal structure of the *Escherichia coli* RND pump AcrAB revealed an unusually large "binding site" capable of accommodating a vast variety of substrates (*17*). At the same time, MDRs have preferences, and the best substrates for all studied MDR groups are hydrophobic cations (*16*, *18*). A positive charge allows a molecule to accumulate in the cell, driven by the transmembrane potential. An ability to accumulate up to 1000-fold makes cations potentially highly toxic to the cells, and this threat may have been responsible for the origin of MDRs. Benzalkonium chloride is an example of a hydrophobic cation that is widely used as an antiseptic and whose efficiency is limited by MDR efflux (*19*). It was recently shown that creating a polymer of the hydrophobic cation hexyl pyridinium makes the compound insensitive to MDR efflux (*20*). Apparently, the pumps can extrude small molecules but not large polymers. This enables creation of effective "sterile surface" materials to prevent the spread of pathogens (*21*). At the same time, it would be very useful to have small molecules in our arsenal of potential pharmaceuticals that avoid MDR efflux. As described below, plants provide a natural example of a chemical strategy to block MDR efflux, thereby allowing antimicrobial compounds synthesized by the plant to inhibit the growth of microbial pathogens.

The alkaloid berberine is a natural product and a hydrophobic cation that is the principal component of the medicinal plants golden seal (*Hydrastis canadensis*) and echinacea (*Echinacea* species). Berberine is a potentially excellent antimicrobial, because it accumulates in cells driven by the membrane potential (*19*) and hits two immutable targets, the membrane and DNA (*22*). Accumulation of hydrophobic cations in the membrane causes leaks, and berberine is also an excellent DNA intercalator (*23*). Resistance to berberine is thus unlikely to develop due to target modification. It was previously shown that resistance to berberine is based on MDRs (*18*); for example, it is readily pumped out of *S. aureus* cells by NorA, an MDR pump responsible for efflux of cationic antiseptics and fluoroquinolones. However, *Berberis* species of plants produce, in addition to berberine, 5′-methoxyhydnocarpin (5-MHC), an inhibitor of MF MDRs. 5-MHC strongly potentiates the action of berberine (*24*). The synergistic combination of an antimicrobial with an MDR inhibitor results in an effective antimicrobial that avoids bacterial resistance.

Not surprisingly, the presence of MDRs has been an important impediment in the development of new synthetic antibiotics. One approach to solving the penetration problem has been to develop MDR inhibitors (*25–27*), similar to the natural strategy that plants use to combat microbial pathogens (*24*). A potential challenge of this approach, however, is to match pharmacokinetics and other properties of two unrelated molecules.

We reasoned that the challenge of developing an efficacious MDR inhibitor could potentially be met by covalently linking an antimicrobial compound with a MDR inhibitor to create a well-penetrating molecule. Here, we report that combining berberine with the MDR inhibitor INF_{55} produces a novel hybrid antibacterial that is insensitive to MDR efflux.

RESULTS AND DISCUSSION

To test the concept of an antimicrobial/MDR inhibitor hybrid, we synthesized a conjugate between berberine, a hydrophobic cation that is an excellent MDR substrate, and INF_{55}, an inhibitor of MF family MDRs (*25*).

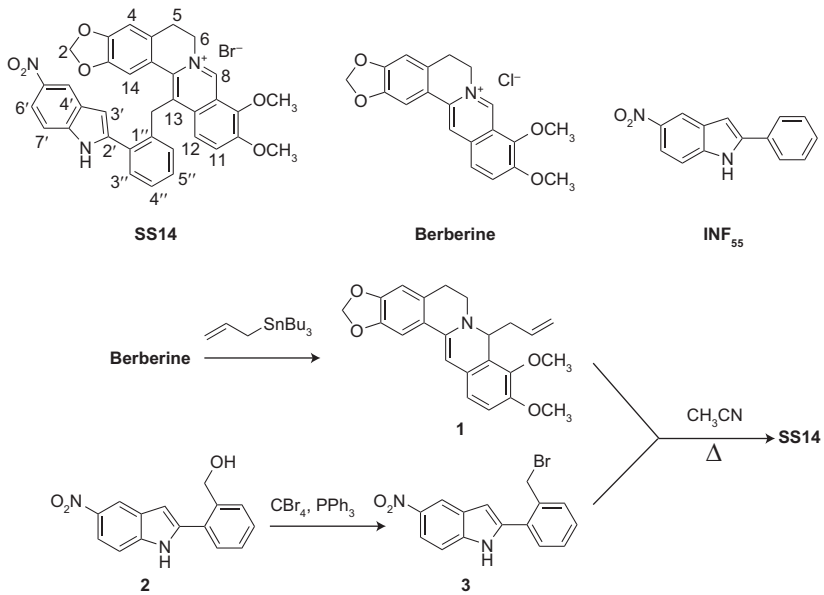

Figure 1. Synthesis of the SS14 hybrid from berberine and INF_{55} derivatives. The hybrid compound SS14 was synthesized in fair yield by reaction of the 8-allyldihydroberberine (1) **with the indole derivative** (3)**. Compound** 1 **was prepared in turn from berberine as described previously, while** 3 **could be accessed from the indole alcohol** (2)**.**

TABLE 1. Minimum inhibitory concentrations of hybrid SS14, berberine, and MDR inhibitor

	MIC (μM)			
	Hybrid	Berberine + INF_{55}[a]	Berberine	INF_{55}
S. aureus				
K1758 Δ*norA*	3.125	6.25	40	>525
8325-4 Wild-type	3.125	12.5	325	>525
K2361 NorA++	6.25	50	>650	>525
K2378 NorA++	3.125	50	>650	>525
En. faecalis				
OG1RF	6.25	50	650	>525
MMH594	6.25	50	>650	>525
V583	6.25	50	>650	>525
En. faecium				
DO	3.125	25	80	>525
B. anthracis				
Sterne	6.25	12.5	325	>525
B. cereus				
569	3.125	12.5	325	>525
T	3.125	12.5	650	>525

[a]Berberine and INF_{55} were added at equimolar concentrations.

Design of the Hybrid Antimicrobial. Berberine and INF_{55} were linked *via* a methylene group (Figure 1). A short linker was chosen in order to minimize any potential problems from increased steric volumes in the hybrid molecule. The hybrid compound, SS14, was synthesized by reaction of 8-allyldihydroberberine (compound **1**) with the indole derivative (compound **3**). Compound **1** was prepared in turn from berberine as described previously (*28*), while compound **3** could be accessed from the indole alcohol (compound **2**) (*29*).

Antibacterial Activity. The resulting conjugate had excellent antimicrobial activity (Table 1). When used against *S. aureus*, SS14 was 100 × more active than berberine. The difference was even greater (200–400 fold) in the case of *S. aureus* mutants overexpressing the NorA MDR. The activity of berberine, as measured by the minimal inhibitory concentration (MIC), against an assortment of tested bacteria varied widely, from 50 to 1300 μM. This is to be expected and likely depends on the level of expression of a variety of MDRs in these organisms. In contrast to berberine, the MIC of the hybrid was essentially the same in all strains tested, 3–6 μM (note that ≤2-fold differences are considered insignificant in the MIC test). *Enterococcus faecalis*, which is known for its high levels of "intrinsic antibiotic resistance" (*30*, *31*), was especially resistant to berberine (MIC 650–1300 μM) but susceptible to the hybrid. The hybrid was also more active than an equimolar combination of berberine and INF_{55} (Table 1). As expected, berberine in the presence of INF_{55} was more effective against a strain lacking the NorA pump as compared with wild-ype and overexpressing mutants. This is apparently due to the presence of additional MDRs in *S. aureus* and is consistent with previous findings from our group (*18*) and from other authors (*32*). By contrast, the activity of the hybrid was not affected by the presence or absence of NorA, apparently indicating that penetration of this molecule was largely unaffected by MDRs.

Similarly to the combination of INF_{55} and berberine, the hybrid had limited activity against Gram-negative species. The MICs were 192 μM against *Pseudomonas aeruginosa*, 96 μM against *E. coli*, and 24 μM against *Salmonella typhimurium*. This is to be expected, since Gram-negative species possess RND MDRs, which are insensitive to INF_{55}. Apparently, the hybrid is unable to bypass RND MDRs as well.

A more detailed examination of growth inhibition of *S. aureus* by the hybrid was made (Figure 2). Measuring inhibition of *S. aureus* growth as a function of concentration shows rapidly increasing inhibition of growth with the hybrid at ~100-fold lower concentration than berberine. A combination of berberine and INF_{55} was less effective than the hybrid. At higher concentrations, INF_{55} showed the paradoxical effect of decreased growth inhibi-

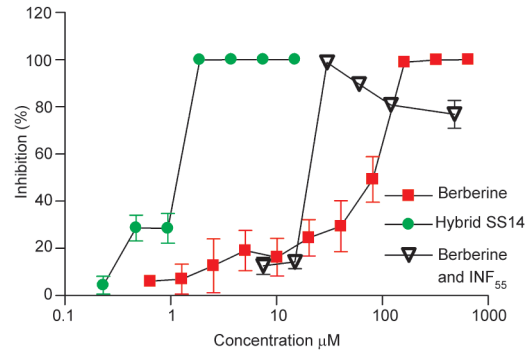

Figure 2. Potentiation of berberine action against *S. aureus* by disabling MDR efflux. Actively growing wild-type *S. aureus* cells were treated with berberine or hybrid SS14. Berberine at a fixed concentration (1.87 μM) was potentiated by varying amounts of INF_{55}.

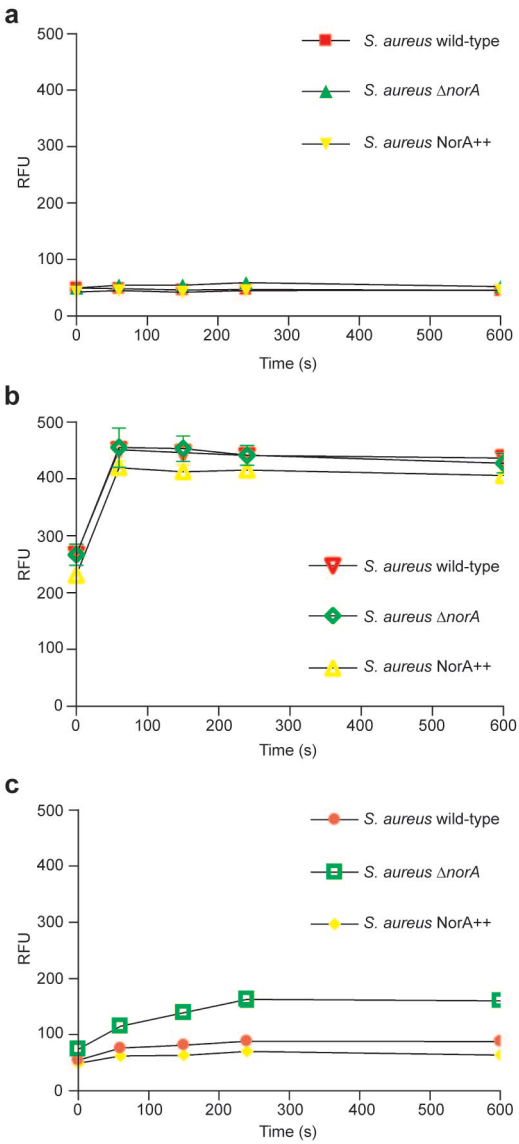

Figure 3. Accumulation of berberine and the hybrid SS14 by *S. aureus*. a) Berberine at 3 μM. b) Hybrid at 3 μM. c) Berberine at 3 μM plus INF_{55} at 3 μM. Accumulation was measured by increase in fluorescence and expressed as relative fluorescence units (RFU).

tion, possibly due to complex formation at high concentrations of INF_{55} and berberine (J. B. Bremner, University of Wollongong, unpublished), which effectively decreases the concentration of free berberine and may decrease its ability to bind DNA, for example. We did not observe a comparable paradoxical effect with SS14. This is perhaps not surprising, given the steric hindrance between the berberine and INF_{55} moieties and the consequent different conformational preferences of the bulky *o*-substituted 2′-aryl group on the indole moiety, which would not apply to the complex of two free molecules. Specifically, we see good DNA interaction for SS14, comparable to that of berberine, as observed by increased fluorescence.

Hybrid Transport. We had previously monitored increase in fluorescence upon accumulation of berberine in cells (*29*). The same approach was used to monitor uptake of SS14 (Figure 3). We chose the MIC concentration of SS14, 3 μM, for this experiment. Rapid accumulation of SS14 was observed with all strains tested (Δ*norA*, wild-type, and *norA*++), whereas there was essentially no accumulation of berberine at the same concentration. This is consistent with the dramatically lower antimicrobial activity of berberine compared with the hybrid. Accumulation of berberine was observed in the presence of INF_{55}, although the rate and level of uptake were lower compared with the hybrid, which also agrees with the antimicrobial activity data. To study efflux in *S. aureus*, we loaded cells with the hybrid or berberine and then transferred them into fresh medium. It was not possible to load the cells, however, using the same concentration of SS14 and berberine. When loaded with berberine at 3 μM, the MIC of the hybrid, there was no noticeable efflux of berberine due to the nominal amount of accumulation. The level of berberine had to be increased substantially in the loading phase to 80 μM in order to observe efflux (Figure 4). At this concentration, SS14 caused rather rapid lysis of cells, which is to be expected, since the membrane is an important site of action of hydrophobic cations, and this concentration is 26-fold above its MIC (a comparable level of berberine would have been 8450 μM, above the limit of solubility). We therefore compared the relative changes in efflux using berberine at 80 μM and the hybrid at 3 μM (Figure 4). Hybrid efflux eventually leveled off, as did berberine efflux in the presence of INF_{55}, whereas berberine efflux continued unabated.

Antibacterial Activity *in Vivo*. To test the potential of the berberine MDR inhibitor conjugate in an *in vivo* model of infection, we took advantage of a pathogenesis model that utilizes the well-studied nematode worm *Caenorhabditis elegans* persistently infected with *En. faecalis* (*33*). We have recently shown that *C. elegans* infected with a variety of human bacterial pathogens that normally kill *C. elegans* can be cured by treatment

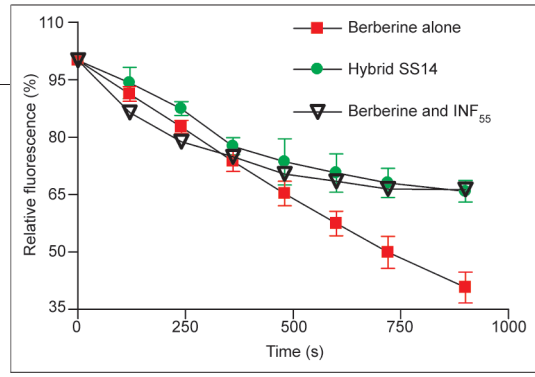

Figure 4. Efflux of berberine and hybrid by *S. aureus norA++*. Cells were loaded with berberine at 80 μM and then resuspended in buffer in the absence of inhibitor or in the presence of inhibitor INF_{55} or loaded with hybrid at 3 μM and resuspended in buffer.

with conventional antibiotics (*34*). *C. elegans* were allowed to ingest cells of *En. faecalis*, which causes a persistent intestinal infection leading to the death of the nematode (*33*). Vancomycin is an effective antibiotic used to treat enterococcal infections, and it produced a substantial curative effect (Figure 5). The hybrid also showed good anti-infective activity. By contrast, berberine alone had no effect, consistent with its low activity against *En. faecalis*. Interestingly, co-administering berberine with the INF_{55} was even worse than the mock-treated control, apparently due to toxicity of INF_{55}. It appears that the hybrid not only improves on the activity of berberine but possibly also cancels the toxicity of the MDR inhibitory moiety. Indeed, INF_{55} was toxic to human cells at 50 μg mL^{-1} (P. Markham, Grants abc, Clifton, VA, personal communication), while SS14 did not show cytotoxicity at >100 μg mL^{-1} (J. B. Bremner, University of Wollongong, unpublished). In summary, these results indicate that conjugating an antimicrobial prone to efflux to an MDR inhibitory moiety can produce an antimicrobial with excellent activity.

Conjugating molecules with different functionalities to produce a hybrid antimicrobial has been reported previously (*35*, *36*), but this work is the first example of an anti-infective/MDR inhibitor conjugate. Future work will examine the detailed mechanism by which this compound penetrates into the cell. In this study, we intentionally chose a preferred MDR substrate, a hydrophobic cation. This suggests that other compounds extruded by MDRs can be similarly potentiated by implementation of the design principle of conjugation to a suitable MDR inhibitor.

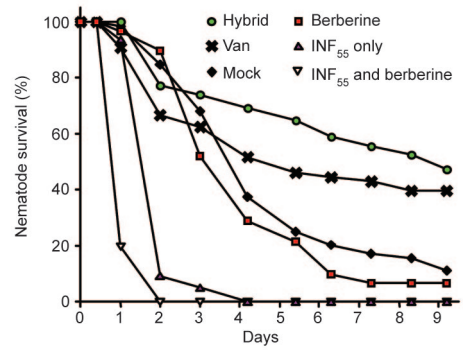

Figure 5. The effect of the hybrid on the survival of nematodes infected with *En. faecalis*. Infected nematodes were mock treated or treated with berberine, INF_{55} only, INF_{55} in combination with berberine, vancomycin, or hybrid. In pairwise comparison log rank tests, the difference in survival curves between mock and hybrid treatments was $p < 0.0001$.

METHODS

Bacterial Strains and Culture Conditions. The following bacterial strains were used in this study: *S. aureus* 8325-4 (wild-type); K1758 (8325-4 Δ*norA*) (*32*); K2361 (K1758/pK364:*norA*, with *norA* from *S. aureus* SA1199B (*37*)); K2378 (K1758/pK374:*norA* with *norA* from *S. aureus* SA1199 (*37*)) (G. Kaatz, VA Medical Center, Detroit, MI, personal communication).

S. aureus was grown in Mueller–Hinton broth, *Bacillus cereus* T and 594 were grown in Luria–Bertani broth, and *En. faecalis* V583, MMH594, and OG1RF were grown in brain–heart infusion (BHI) broth. Growth of K1758 was supplemented with erythromycin (20 μg mL^{-1}) and that of K2361 and K2378 with chloramphenicol (20 μg mL^{-1}).

Chemicals and Antibiotics. Berberine chloride, chloramphenicol, and erythromycin were purchased from Sigma Chemical Co. INF_{55} and INF_{271} were purchased from ChemBridge. Compound SS14 was determined to be >95% pure by HPLC analysis (Phenomenex Luna C18 (5 μm) column, 150 mm × 4.60 mm; UV detection, 254 nm; gradient elution, solvent A = 100% H_2O (0.1% conc HCl) and solvent B = 10% H_2O, 90% CH_3CN (0.1% conc HCl); gradient timing: 0 min 70% solvent A, 30% solvent B; 2 min 70% solvent A, 30% solvent B; 32 min 0% solvent A, 100% solvent B; R_t = 24.7 min) and by ^1H NMR spectroscopy; commercial compounds were also noted as >95% pure.

Antimicrobial Susceptibility. Cells (10^5 mL^{-1}) were inoculated into broth and dispensed at 0.2 mL $well^{-1}$ in 96-well microtiter plates. MICs were determined in triplicate by serial 2-fold dilution of the test compound. The MIC was defined as the concentration of an antimicrobial that completely inhibited cell growth during an 18 h incubation at 37 °C or 20 h incubation at 30 °C. Growth was assayed with a microtiter plate reader (Spectramax PLUS384; Molecular Devices) by monitoring absorption at 600 nm.

Uptake Assay. *S. aureus* was cultured with aeration at 37 °C to an optical density of 1.5 at 600 nm (OD_{600}), pelleted by centrifu-

gation for 2 min at 12,000 RPM, and washed twice with 25 mM phosphate-buffered saline (PBS). Cells were then resuspended to an OD_{600} of 0.8 in PBS buffer containing 10 mM dextrose and incubated for 1 h at 37 °C with aeration. Cells were washed twice by centrifugation for 2 min at 12,000 RPM with PBS containing dextrose. Cells were then resuspended to an OD_{600} of 0.3 in PBS. Assays were performed in 96-well flat-bottom white microtiter plates (NUNC) in a final volume of 200 μL. Hybrid was added at 3 μM, berberine at 3 μM, and INF_{55}, when present, at 3 μM. Fluorescence was measured with a Spectramax GeminiXS spectrofluorometer (Molecular Devices) at a 355-nm excitation wavelength and a 517-nm emission wavelength. Both SS14 and berberine showed low and equivalent background fluorescence, which was blanked, in the absence of cells.

Efflux Assay. *S. aureus norA++* was cultured with aeration at 37 °C to an OD_{600} between 0.9 and 1, pelleted by centrifugation for 2 min at 12,000 RPM, and then washed and resuspended in 25 mM PBS (pH 7.3) containing 0.05g L^{-1} $MgSO_4$, 7g L^{-1} K_2HPO_4, 0.5 g L^{-1} sodium citrate·$3H_2O$, 1g L^{-1} $(NH_4)_2SO_4$, 0.01 mg L^{-1} folic acid, 0.05 mg L^{-1} pyridoxine hydrochloride, 0.025 mg L^{-1} riboflavin, 0.01 mg L^{-1} biotin, 0.025 mg L^{-1} thiamine, 0.025 mg L^{-1} nicotinic acid, 0.025 mg L^{-1} calcium pantothenate, 0.5 μg L^{-1} vitamin B12, 0.025 mg L^{-1} *p*-aminobenzoic acid, 0.025 mg L^{-1} thiotic acid, and 4.5 mg L^{-1} monopotassium phosphate. Cells were then resuspended to an OD_{600} of 0.8 in buffer with 10 mM dextrose. Cells were then loaded with either 80 μM berberine and 30 μg mL^{-1} reserpine or 3 μM SS14 and incubated at 37 °C with aeration for 20 min. Cells were then centrifuged for 2 min at 12,000 RPM in a 4 °C cold room, washed in ice-cold PBS, and added at an OD_{600} of 0.3 to a chilled 96-well flat-bottom white microtiter plate (NUNC) containing ice-cold 25 mM PBS and 10 mM dextrose in a final volume of 200 μL. Some wells contained INF_{55} at 5 μg mL^{-1}. Fluorescence was measured with a Spectramax GeminiXS spectrofluorometer (Molecular Devices) at a 355-nm excitation wavelength and a 517-nm emission wavelength.

Nematode Curing Assay. *C. elegans* strain *glp-4(bn2ts);sek-1(km4)*, which is a temperature-sensitive sterile mutant that has enhanced susceptibility to pathogens (*38*), was synchronized by isolating eggs and hatching them overnight in M9 buffer. The L1 stage nematodes were grown on *E. coli* strain OP50 on NGM agar media at 25 °C to the young adult stage. Nematodes were suspended in M9 buffer and transferred onto a lawn of *En. faecalis* strain OG1RF on BHI agar containing 80 μg mL^{-1} kanamycin to inhibit *E. coli* growth. Nematodes were infected for 9 h at 25 °C and resuspended with M9 buffer, and then 30–40 worms were pipetted onto 35 mm Petri plates containing 3 mL of BHI agar plus the appropriate compounds: the test compounds were incorporated into BHI media at 75 μM and vancomycin at 25 μg mL^{-1}. The BHI media for all of the samples included 1% DMSO, 3% ethanol, and 80 μg mL^{-1} kanamycin. Worm survival was monitored, and worms were considered to be dead when they were unresponsive to touch. Statistical analysis was performed according to the method of Kaplan–Meier.

Synthesis of Compound 3 and SS14. For general experimental details, see ref *29*.

2-(2-Bromomethyl-phenyl)-5-nitro-1*H*-indole (3). A suspension of the alcohol **2** (*29*) (200 mg, 0.75 mmol), triphenylphosphine (390 mg, 1.5 mmol), and carbon tetrabromide (490 mg, 1.5 mmol) in dry diethyl ether (60 mL) was stirred with warming at 40 °C under a nitrogen atmosphere for 2 d. The reaction mixture was then filtered, and the filtrate was concentrated. The residual yellow oil was chromatographed on silica gel by vacuum liquid chromatography (silica gel, 20% EtOAc in petroleum spirit, bp 40–60 °C) to give **3** (102.3 mg, 41%) as a yellow solid, mp 164–166 °C. ^{1}H NMR (300 MHz, $CDCl_3$,): δ 4.64 (s, 2H, CH_2Br), 6.93 (d, *J* = 1.2 Hz, 1H, H-3), 7.43–7.58 (m, 5H, H-7, aromatic), 8.16 (dd, *J* = 9.0, 2.1 Hz, H-6), 8.64 (d, *J* = 2.1 Hz, 1H, H-4), 9.14 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 33.0 (CH_2Br), 105.1 (C3), 111.1 (C7), 117.8 (C4), 118.6 (C6), 127.9 (C3a), 129.4 (aromatic), 129.6 (aromatic), 130.5 (aromatic), 131.7 (aromatic), 131.8 (C2), 135.6 (C1′), 139.3 (C7a)[a], 139.6 (C2′)[a], 142.3 (C5). Superscript letters in NMR data indicate interchangeable assignments. High-resolution mass spectrometry (HRMS) (electron ionization (EI)): *m/z* calcd for $C_{15}H_{11}N_2O_2{}^{79}Br$ $[M]^+$, 330.0003; found, 329.9982.

9,10-Dimethoxy-13-[2-(5-nitro-1*H*-indol-2-yl)benzyl]-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium Bromide, SS14. A solution of the dihydroberberine **1** (*28*) (91.0 mg, 0.24 mmol) and the benzyl bromide **3** (102.3 mg, 0.30 mmol) in dry CH_3CN (7 mL) was heated at reflux for 24 h under a nitrogen atmosphere. The mixture was then concentrated and triturated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The solid was chromatographed on silica gel (6% MeOH in dichloromethane) to give **SS14** (55.0 mg, 35%) as a yellow solid; mp 206 °C (dec.). ^{1}H NMR (300 MHz, CD_3OD): δ 3.03 (t, *J* = 5.5 Hz, 2H, H-5), 4.01 (s, 3H, OCH_3), 4.17 (s, 3H, OCH_3), 4.80 (br.s, 2H, H-6), 4.84 (s, 2H, CH_2Ph), 5.96 (s, 2H, OCH_2O), 6.72 (s, 1H, H3′), 6.86 (s, 1H, H-4)[a], 6.90 (s, 1H, H-14)[a], 6.96 (d, *J* = 7.8 Hz, 1H, H-6″), 7.27 (td, *J* = 7.7, 1.5 Hz, 1H, H-5″), 7.37 (br.t, *J* = 7.5 Hz, 1H, H-4″), 7.42 (d, *J* = 9.0 Hz, 1H, H-7′), 7.58 (dd, *J* = 7.7, 1.1 Hz, 1H, H-3″), 7.78 (d, *J* = 9.3 Hz, 1H, H-11)[b], 7.88 (dd, *J* = 9.0, 2.4 Hz, 1H, H-6′), 7.94 (d, *J* = 9.3 Hz, 1H, H-12)[b], 8.34 (d, *J* = 2.1 Hz, 1H, H-4′), 9.8 (s, 1H, H-8). ^{13}C NMR (75 MHz, CD_3OD): δ 29.1 (C5), 36.4 (CH_2Ar), 57.5 (OCH_3), 58.8 (C6), 62.7 (OCH_3), 103.6 (OCH_2O), 105.6 (C3′), 109.3 (C4)[c], 109.8 (C14)[c], 112.3 (C7′), 118.2 (C6′), 118.3 (C4′), 121.4 (C4a), 122.5 (C11)[d], 122.9 (C8a)[e], 127.3 (C12)[d], 128.6 (C4″), 129.2 (C3a′), 130.4 (C6″, C5″), 131.8 (C3″), 132.8 (C12a)[e], 133.4 (C2′), 135.0 (C13b), 135.1 (C13), 138.6 (C1″)[f], 139.0 (C13a), 141.2 (C7a′), 141.5 (C2″)[f], 142.9 (C5′), 146.1 (C8), 146.2 (C9)[g], 148.6 (C3a)[h], 151.4 (C14a)[h], 151.7 (C10)[g]. HRMS (electrospray): *m/z* calcd for $C_{35}H_{28}N_3O_6$ $[M]^+$, 586.1978; found, 586.1984.

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REFERENCES

1. Lewis, K., and Lomovskaya, O. (2002) in *Bacterial Resistance to Antimicrobials: Mechanisms, Genetics, Medical Practice and Public Health* (Lewis, K., Salyers A., Taber H., and Wax, R., Eds.) pp 6190, Marcel Dekker, New York.
2. Levy, S. B. (2002) The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance, *J. Antimicrob. Chemother. 49*, 25–30.
3. Li, X. Z., and Nikaido, H. (2004) Efflux-mediated drug resistance in bacteria, *Drugs 64*, 159–204.
4. Poole, K. (2005) Efflux-mediated antimicrobial resistance, *J. Antimicrob. Chemother. 56*, 20–51.
5. Saier, M. H., Jr. , and Paulsen, I. T. (2001) Phylogeny of multidrug transporters, *Semin. Cell Dev. Biol. 12*, 205–213.
6. Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells, *Cell 47*, 381–389.
7. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1989) Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: Evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein, *J. Histochem. Cytochem. 37*, 159–164.

8. Schinkel, A. H., Smit, J. J. M., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P. J., Berns, A. J. M., and Borst, P. (1994) Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs, *Cell 77*, 491–502.
9. Loscher, W., and Potschka, H. (2005) Drug resistance in brain diseases and the role of drug efflux transporters, *Nat. Rev. Neurosci. 6*, 591–602.
10. Gottesman, M. M., Fojo, T., and Bates, S. E. (2002) Multidrug resistance in cancer: role of ATP-dependent transporters, *Nat. Rev. Cancer 2*, 48–58.
11. Konings, W. N., and Poelarends, G. J. (2002) Bacterial multidrug resistance mediated by a homologue of the human multidrug transporter P-glycoprotein, *IUBMB Life 53*, 213–218.
12. Zgurskaya, H. I., and Nikaido, H. (2000) Multidrug resistance mechanisms: drug efflux across two membranes, *Mol. Microbiol. 37*, 219–225.
13. Markham, P. N., and Neyfakh, A. A. (2001) Efflux-mediated drug resistance in Gram-positive bacteria, *Curr. Opin. Microbiol. 4*, 509–514.
14. Paulsen, I. T., Brown, M. H., and Skurray, R. A. (1998) Characterization of the earliest known *Staphylococcus aureus* plasmid encoding a multidrug efflux system, *J. Bacteriol. 180*, 3477–3479.
15. Gros, P., Talbot, F., Tang-Wai, D., Bibi, E., and Kaback, H. R. (1992) Lipophilic cations: a group of model substrates for the multidrug-resistance transporter, *Biochemistry 31*, 1992–1998.
16. Lewis, K. (2001) In search of natural substrates and inhibitors of MDR pumps, *J. Mol. Microbiol. Biotechnol. 3*, 247–254.
17. Yu, E. W., McDermott, G., Zgurskaya, H. I., Nikaido, H., and Koshland, D. E., Jr. (2003) Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump, *Science 300*, 976–980.
18. Hsieh, P. C., Siegel, S. A., Rogers, B., Davis, D., and Lewis, K. (1998) Bacteria lacking a multidrug pump: A sensitive tool for drug discovery, *Proc. Natl. Acad. Sci. U.S.A. 95*, 6602–6606.
19. Severina, I. I., Muntyan, M. S., Lewis, K., and Skulachev, V. P. (2001) Transfer of cationic antibacterial agents berberine, palmatine and benzalkonium through bimolecular planar phospholipid film and *Staphylococcus aureus* membrane, *IUBMB Life Sci. 52*, 321–324.
20. Lin, J., Tiller, J. C., Lee, S. B., Lewis, K., and Klibanov, A. M. (2002) Insights into bactericidal action of surface-attached poly(vinyl-N-hexylpyridinium) chains, *Biotechnol. Lett. 24*, 801–805.
21. Lewis, K., and Klibanov, A. M. (2005) Surpassing nature: rational design of sterile-surface materials, *Trends Biotechnol. 23*, 343–348.
22. Amin, A. H., Subbaiah, T. V., and Abbasi, K. M. (1969) Berberine sulfate: antimicrobial activity, bioassay, and mode of action, *Can. J. Microbiol. 15*, 1067–1076.
23. Davidson, M. W., Lopp, I., Alexander, S., and Wilson, W. D. (1977) The interaction of plant alkaloids with DNA. II. Berberinium chloride, *Nucleic Acids Res. 4*, 2697–2712.
24. Stermitz, F. R., Lorenz, P., Tawara, J. N., Zenewicz, L., and Lewis, K. (2000) Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor, *Proc. Natl. Acad. Sci. U.S.A. 97*, 1433–1437.
25. Markham, P. N., Westhaus, E., Klyachko, K., Johnson, M. E., and Neyfakh, A. A. (1999) Multiple novel inhibitors of the NorA multidrug transporter of Staphylococcus aureus, *Antimicrob. Agents Chemother. 43*, 2404–2408.
26. Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S., Renau, T., Leger, R., Hecker, S., Watkins, W., Hoshino, K., Ishida, H., Lee, V. J. (2001) Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: Novel agents for combination therapy, *Antimicrob. Agents Chemother. 45*, 105–116.
27. Lomovskaya, O., and Bostian, K. A. (2006) Practical applications and feasibility of efflux pump inhibitors in the clinic—a vision for applied use, *Biochem. Pharmacol. 71*, 910–918.
28. Bremner, J.B., and Samosorn, S. (2003) 8-Allydihydroberberine as an alternative precursor for the synthesis of 13-substituted berberine derivatives, *Austr. J. Chem. 56*, 871–873.
29. Samosorn, S., Bremner, J. B., Ball, A., and Lewis, K. (2006) Synthesis of functionalized 2-aryl-5-nitro-1 *H*-indoles and their activity as bacterial NorA efflux pump inhibitors, *Bioorg. Med. Chem. 14*, 857–865.
30. Lynch, C., Courvalin, P., and Nikaido, H. (1997) Active efflux of antimicrobial agents in wild-type strains of enterococci, *Antimicrob. Agents Chemother. 41*, 869–871.
31. Gilmore, M. S., Sahm, D. F., and Huycke, M. M. (1998) Multi-drug resistant enterococci: The nature of the problem and an agenda for the future, *Emerging Infect. Dis. 4*, 239–249.
32. Price, C. T., Kaatz, G. W., and Gustafson, J. E. (2002) The multidrug efflux pump NorA is not required for salicylate-induced reduction in drug accumulation by Staphylococcus aureus, *Int. J. Antimicrob. Agents 20*, 206–213.
33. Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., Calderwood, S. B., and Ausubel, F. M. (2001) A simple model host for identifying Gram-positive virulence factors, *Proc. Natl. Acad. Sci. U.S.A. 98*, 10892–10897.
34. Moy, T. I., Ball, A. R., Anklesaria, Z., Casadei, G., Lewis, K., and Ausubel, F. M. (2006) Identification of novel antimicrobials using a live-animal infection model, *Proc. Natl. Acad. Sci. U.S.A. 103*, 10414–10419.
35. Tegos, G. P., Anbe, M., Yang, C., Demidova, T. N., Satti, M., Mroz, P., Janjua, S., Gad, F., and Hamblin, M. R. (2006) Protease-stable polycationic photosensitizer conjugates between polyethyleneimine and chlorin(e6) for broad-spectrum antimicrobial photoinactivation, *Antimicrob. Agents Chemother. 50*, 1402–1410.
36. Merrifield, R. B., Juvvadi, P., Andreu, D., Ubach, J., Boman, A., Boman, H. G. (1995) Retro and retroenantio analogs of cecropin–melittin hybrids, *Proc. Natl. Acad. Sci. U.S.A. 92*, 3449–3453.
37. Kaatz, G. W., and Seo, S. M. (1997) Mechanisms of fluoroquinolone resistance in genetically related strains of Staphylococcus aureus, *Antimicrob. Agents Chemother. 41*, 2733–2737.
38. Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M. W., and Ausubel, F. M. (2002) A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity, *Science 297*, 623–626.