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Symmetric Bis-benzimidazoles Are Potent Anti-Staphylococcal Agents with Dual Inhibitory Mechanisms against DNA Gyrase

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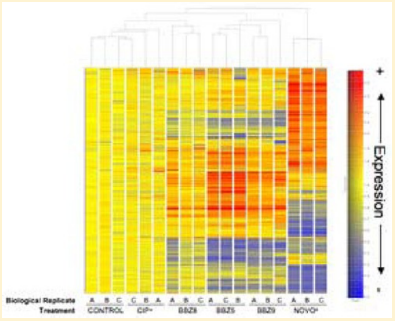
Supporting Information

ABSTRACT: Various bis-benzimidazole derivatives have been reported to possess activity against Gram-positive pathogens. No mechanism of action has been elucidated to fully account for the antibacterial activity of this class of compounds. A group of symmetric bis-benzimidazoles (BBZ) designed as anticancer agents have previously been shown to possess moderate antiproliferative activity. We sought to assess the antibacterial activity and mechanism of action of BBZ compounds against *Staphylococcus aureus*. Antibacterial activities were assessed by determination of minimal inhibitory concentrations (MICs), time-kill curves, and scanning electron microscopy. Transcriptional responses to BBZ treatment were determined using whole genome microarrays. Activities against bacterial type II topoisomerases were investigated using in vitro supercoiling, decatenation, DNA binding, and DNA cleavage inhibition assays. MICs for EMRSA-16 were between 0.03 and 0.5 μg/mL. The compounds showed concentration-dependent bactericidal activity and induced cell swelling and lysis. Transcriptional responses to BBZ were consistent with topoisomerase inhibition and DNA damage. A subset of BBZ compounds inhibited *S. aureus* DNA gyrase supercoiling activity with IC_{50} values in the range of 5−10 μM. This inhibition was subsequently shown to operate through both inhibition of binding of DNA gyrase to DNA and accumulation of single-stranded DNA breaks. We conclude that BBZ compounds are potent anti-staphylococcal agents and operate at least in part through DNA gyrase inhibition, leading to the accumulation of single-stranded DNA breaks, and by preventing the binding of gyrase to DNA.



With the continuing emergence of clinically important multidrug-resistant pathogenic bacteria, there is an ongoing need for new antibacterial agents.[1,2] In the past decade, there has been a resurgence of interest in benzimidazole-containing compounds for use as antibacterial agents, in particular against Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA). Recently reported compounds include dicationic bis-benzimidazoles with reasonable anti-MRSA activities and aminobenzimidazoles with potent activity against a wide range of bacterial pathogens.[3−5] The latter are inhibitors of the bacterial type II topoisomerases.[6] A series of pyrimido[1,6-α]benzimidazoles have also been reported to be inhibitors of bacterial DNA gyrase that possess antibacterial activity against Gram-negative and Gram-positive species.[7,8] However, no extensive studies of the molecular mechanisms of this class of compounds have been reported.

The bacterial topoisomerases are essential for cell survival and represent an example of an established drug target for which novel mechanisms of inhibition are very attractive.[9] Bacterial genomes typically encode two type II topoisomerases, DNA gyrase and topoisomerase IV (topo IV). Both play crucial roles in relieving the topological stresses associated with DNA replication, translation, and chromosome segregation.[10] DNA gyrase and topo IV act in a similar manner, binding DNA and introducing a pair of single-stranded breaks, creating a DNA "gate" through which another DNA strand can be threaded. For gyrase, which acts ahead of the replication fork, this action allows the introduction of negative supercoils into the proceeding duplex. Topo IV, in contrast, acts primarily as a decatenating enzyme to facilitate the untangling of daughter chromosomes following replication. Both enzymes are heterotetramers, composed of two identical subunits each of GyrA and GyrB (gyrase) or GrlA and GrlB (topo IV). The GyrA and GrlA subunits are largely responsible for DNA binding, breakage, and resealing, and the GyrB and GrlB subunits provide the energy for the reaction through ATP binding and hydrolysis.[11]

Bacterial topoisomerases are inhibited by several distinct groups of antibacterials.[9] One major group is the quinolones, which include ciprofloxacin. Quinolones act through formation of a ternary complex with the DNA and the GyrA and GrlA subunits of the topoisomerase, which is trapped at the "open gate" stage. This presents free DNA ends that, when released from the complex, create the equivalent of double-stranded breaks, leading to the accumulation of reactive oxygen species

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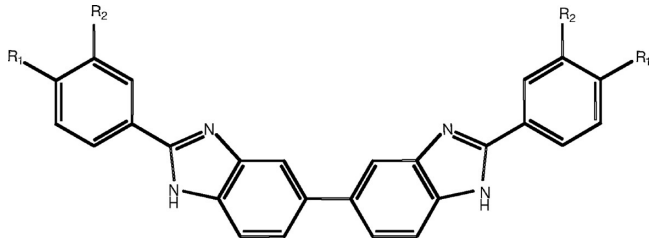
Table 1. In Vitro Activities of Symmetric Bis-benzimidazoles against *S. aureus* and Cytotoxicities in a Human Cell Line



			MIC (μg/mL)[a]			IC_{50} (μM)[b]
			S. aureus			*Homo sapiens*
Compound	R_1	R_2	ATCC 29213	NCTC 8325	EMRSA-16	WI-38
BBZ1	CH_3	H	>128	>16	>128	>32
BBZ2	CH_2CH_3	H	>128	>16	>128	1.84 ± 0.64
BBZ3	CF_3	H	128	>16	64	0.31 ± 0.07
BBZ4	OCH_2CH_3	H	0.06	0.06	0.125	4.00 ± 1.31
BBZ5	NH_2	H	0.125	0.125	0.5	9.83 ± 0.49
BBZ6	Cl	H	8	4−8	>128	>64
BBZ7	F	H	>128	>16	>128	>64
BBZ8	H	OCH_3	>128	>16	>128	37.33 ± 4.62
BBZ9	OCH_3	H	0.03−0.06	0.03	0.06	2.80 ± 0.16
BBZ10	OCH_2Bn	H	>128	>16	>128	>64
novobiocin			0.06	2	0.5	1.40
ciprofloxacin			0.25	0.125	>16	1.16

[a]Values provide the range of MIC values observed for at least three biological replicates. [b]Values are the means of four biological replicates ± the standard deviation where indicated.

and subsequent cell death.[12,13] A second group, the coumarins (e.g., novobiocin), target topoisomerases through the GyrB and GrlB subunits, competing for ATP and thus inhibiting their enzymatic activities.[11] In addition, two new classes of topoisomerase inhibitors with novel modes of action have recently been described. The first of these, the simocyclinones, have been shown to inhibit DNA gyrase-mediated DNA supercoiling and relaxation by preventing the binding of the enzyme to DNA.[14] The second recently described group of novel bacterial topoisomerase inhibitors are quiniline derivatives that inhibit DNA gyrase by stabilizing a precleavage intermediate, resulting in the accumulation of single-stranded DNA breaks.[15]

We reported some years ago on a novel series of symmetric bis-benzimidazoles, including uncharged compounds BBZ4 and BBZ9 (Table 1).[16,17] A disclosure within the patent literature describes antibacterial activity for these uncharged compounds against a number of species, including *Enterococcus* sp. and *Helicobacter pylori*.[18] However, no studies of the mechanism of action have been reported. The symmetric bis-benzimidazoles (BBZ) are based on the cell stain Hoechst 33258, a head-to-tail bis-benzimidazole compound that binds the minor groove of B form DNA at three consecutive A·T base pairs.[19,20] In Hoechst 33258, this interaction disrupts the binding of TATA-box transcription factors to DNA and is thought to be responsible for its inhibitory activity against mammalian DNA topoisomerase I.[21,22] The symmetric head-to-head arrangement of the benzimidazole groups in the BBZ series extends the binding site to four A·T base pairs and confers on some of these compounds moderate antiproliferative and antitumor activity.[23]

This study significantly improves our knowledge of the therapeutic potential and mechanistic activity of antibacterial benzimidazole-containing compounds. We found that a subset of symmetric bis-benzimidazoles possess potent antibacterial activity against *S. aureus*, including the clinical isolate EMRSA-16. Using whole genome microarrays, the gene expression responses to BBZ treatment were characterized in *S. aureus*. The transcriptional profiles indicated both DNA damage and type II topoisomerase inhibition. Subsequent analysis using a series of gyrase inhibition assays provided strong evidence that this antibacterial activity stems from an inhibition of DNA gyrase that leads to the subsequent accumulation of single-stranded DNA breaks. In addition, uncharged compounds within the series were found to inhibit the binding of DNA gyrase to a DNA substrate. We conclude that the BBZ compounds possess two gyrase inhibitory mechanisms that are shared with two structurally distinct series of topoisomerase inhibitors, the simocyclinones and the quiniline derivatives.

EXPERIMENTAL PROCEDURES

Chemicals. BBZ compounds were synthesized at Queen's University (Belfast, Northern Ireland) using previously published synthetic strategies and were analytically pure.[23] Compounds were provided in powdered form, dissolved in DMSO to concentrations of 3.2 mg/mL, and stored at −20 °C. Novobiocin was purchased from Merck and dissolved in high-performance liquid chromatography (HPLC) grade water. Ciprofloxacin was purchased from Sigma (Poole, U.K.) and dissolved in 1 mM HCl (pH 3). Both reference antibiotics were sterilized by filtration through Millex 0.45 μM pore size filter units (Millipore, Cork, Ireland) and stored at 4 °C before being used.

Bacterial Strains and Growth Conditions. *S. aureus* ATCC 29213 and NCTC 8325 are wild-type reference strains obtained from the American Type Culture Collection (ATCC, Manassas, VA) and the National Collection of Type Cultures (NCTC, Salisbury, U.K.), respectively. EMRSA-16 is a clinical isolate from the Royal Free Hospital (London, U.K.). All strains

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were maintained on Mueller-Hinton (MH) Agar (Oxoid, Basingstoke, U.K.) and routinely grown in MH broth with shaking (37 °C and 200 rpm). Overnight cultures (10 mL) were used to inoculate 10 mL test cultures to a starting optical density at 595 nm (OD_{595}) of 0.05. Growth of bacterial cultures was measured OD_{595} and by viable cell counts following serial dilution in PBS and growth on MH agar (37 °C for 16 h).

Antibacterial Susceptibility and Mammalian Cytotoxicity Testing. MICs were determined by the broth dilution method according to published guidelines.[24] MIC values were taken as the lowest concentration for which no visible growth was seen following a 20 h incubation at 37 °C. The cytotoxicity of the BBZ drug panel was assessed in human lung fibroblast cell line WI-38 (ATCC) using the sulforhodamine B (SRB) assay as described previously.[25] The 50% inhibitory concentration (IC_{50}) was taken as the concentration for which the SRB absorbance at 540 nm was 50% of the no drug control.

Scanning Electron Microscopy. DMSO or BBZ compounds were added to bacterial cultures immediately after inoculation. Cells were incubated for 8 or 24 h, collected (1 mL), and washed three times in sterile PBS. Cells were fixed in 2% glutaraldehyde (16 h at room temperature) and then dehydrated by being washed in increasing concentrations of HPLC grade ethanol (30, 50, 70, and 100%). For all wash steps, cells were collected by centrifugation (3000*g* for 10 min). Dehydrated samples were coated with 5 nm gold using a Q150 sputter coater (Quorum) and imaged using an FEI Quanta 200 FEG scanning electron microscope (Eindhoven, The Netherlands).

Bacterial Growth and Treatment for Transcriptional Analysis. Test compounds were added to duplicate 50 mL cultures of *S. aureus* at the midlog stage of growth (2−3 h; $OD_{595} \sim 0.5$−0.6). EMRSA-16 was treated with BBZ5 at 2 μg/mL, and for NCTC 8325, test compounds were added at the following concentrations: BBZ5, 0.5 μg/mL; BBZ8 and BBZ9, 0.216 μg/mL; ciprofloxacin, 0.188 μg/mL; novobiocin, 3.44 μg/mL. For control cultures, the equivalent volume of DMSO solvent was added. Cells were grown for an additional 15 min, at which point half the volume of each duplicate culture was pooled for each treatment and collected by centrifugation (5000*g* for 10 min at 4 °C). Cells were resuspended in 1 mL of MH broth, and the RNA expression profile was stabilized by the addition of 2 mL of RNAProtect reagent (Qiagen) for 5 min at room temperature. Cells were collected by centrifugation (5000*g* for 10 min), and cell pellets were stored at −80 °C. The remaining half culture volumes were grown for an additional 20 h to confirm the inhibitory effects of the compounds. Each treatment was conducted three times on separate days.

Nucleic Acid Preparations and Microarray Analysis. Total RNA was isolated from bacterial pellets as described previously.[26] Genomic DNA was isolated from stationary phase cells pooled from each untreated biological replicate using the Q-100 genomic tip kit (Qiagen), according to the manufacturer's recommendations. For each treatment and replicate, 5 μg of RNA and 2 μg of DNA were labeled with Cy5 and Cy3 dCTP, respectively, using random primers with either Superscript II reverse transcriptase for RNA (Amersham) or Klenow DNA polymerase for DNA (Invitrogen). Fluorescently labeled cDNAs from RNA and gDNA were pooled, purified, and cohybridized onto BμG@S SAv1.1.0 microarrays provided by the Bacterial Microarray Group at St. George's, University of London. Washing, scanning, and feature extraction procedures have been described previously.[27] Array design is available in BμG@Sbase [accession number A-BUGS-17 (http://bugs.sgul.ac.uk/A-BUGS-17)] and also ArrayExpress (accession number A-BUGS-17). Statistical analyses were performed using GeneSpring version 7.3.1 (Agilent Technologies). Differentially expressed genes were defined as those that showed >2-fold up- or downregulation compared to untreated controls, with a *P* value of <0.05 determined by one-way analysis of variance (ANOVA) with Benjamini and Hochberg false discovery rate correction. Fully annotated microarray data have been deposited in BμG@Sbase [accession number E-BUGS-106 (http://bugs.sgul.ac.uk/E-BUGS-106)] and also ArrayExpress (accession number E-BUGS-106).

Quantitative Real-Time Polymerase Chain Reaction. Genes of interest were quantified using two-step, quantitative real-time PCR (qRTPCR). Total RNA samples used in the NCTC 8325 microarray analysis (1 μg) were converted to cDNA, and qPCR was performed using SYBR Green in a Stratagene MX-3000P instrument. Primers were designed using Primer3Plus[28] and are listed in Table S3 of the Supporting Information. Triplicate biological samples were measured in duplicate for each gene, and then the extent of regulation compared to the control was determined using Relative Expression Software Tool-384, version 2.[29] Samples were normalized to *odhB* because of its constant expression across all conditions on the microarray.

Topoisomerase Inhibition Assays. Assessments of topoisomerase inhibition were performed using enzymes and reagents purchased from Inspiralis Ltd. (Norwich, U.K.). All reactions consisted of enzymes (1 unit, activity defined by the supplier) and substrates incubated in the appropriate reaction buffer for 30 min at 37 °C in a total reaction volume of 20 μL. Increasing dilutions of ciprofloxacin, novobiocin, BBZ1−10, or equivalent volumes of solvent were included in test reactions. The maximal amount of DMSO in any test reaction was 2.5% (v/v). Ciprofloxacin and novobiocin were diluted in ddH_2O.

For supercoiling inhibition assays, *S. aureus* DNA gyrase was incubated with relaxed pBR322 in supercoiling assay buffer [5 mM HEPES-KOH (pH 7.6), 2 mM magnesium acetate, 2 mM DTT, 0.4 mM ATP, 100 mM potassium glutamate, and 0.01 mg/mL albumin]. For decatenation inhibition assays, *S. aureus* topo IV was incubated with 100 ng of kDNA in topo IV assay buffer [10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, 1.5 mM ATP, 70 mM potassium glutamate, and 10 μg/mL albumin]. Reactions were stopped by the addition of 20 μL of 2× STEB [40% (w/v) sucrose, 100 mM Tris-HCl (pH 8), 10 mM EDTA, and 0.5 mg/mL bromophenol blue] and 4 μL of 6× blue-orange loading dye. Supercoiling reaction products were loaded directly onto 0.8% TBE-agarose gels and separated by electrophoresis (50 V for 4 h). Decatenation products were loaded directly onto 1% TBE-agarose gels and separated by electrophoresis (80 V for 1 h).

Gels were stained by overnight incubation in TBE buffer containing 0.2 μg/mL ethidium bromide and visualized by fluorescence using a GeneGenius gel imager with GeneSnap acquisition software (Syngene, Cambridge, U.K.). For IC_{50} determinations, the fluorescence intensity of the supercoiled or decatenated bands was quantified using GeneTools analysis software (Syngene). The IC_{50} value was taken as the concentration of the test compound that resulted in a 50% reduction in product band intensity compared to the no drug control.

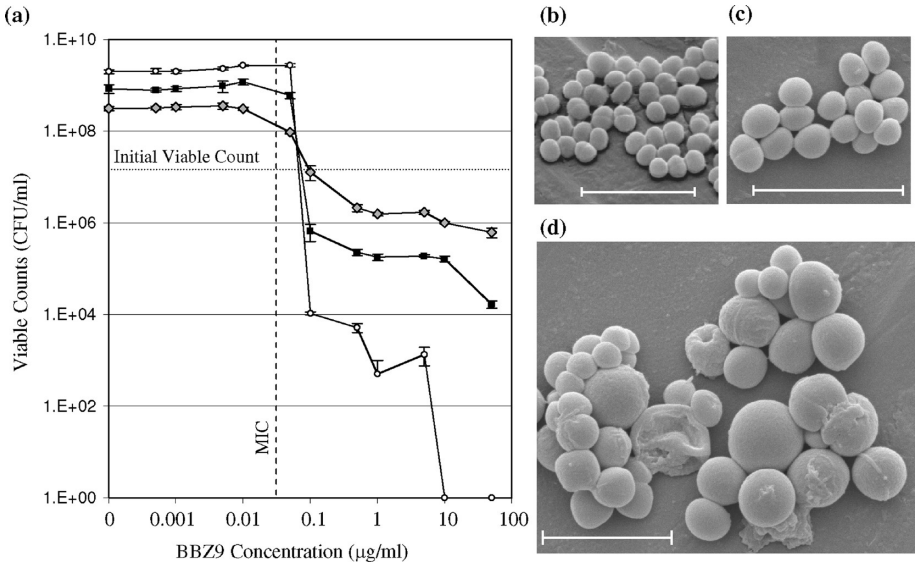

Figure 1. BBZ compounds induce cell swelling and lysis in *S. aureus* leading to bactericidal effects. Shown are the effects of BBZ compounds on *S. aureus* viability and morphology measured by bactericidal profiling (time-kill kinetics) and scanning electron microscopy. (a) Representative bactericidal profile. *S. aureus* NCTC 8325 was grown in the presence of BBZ9 at concentrations ranging from 0 to 50 μg/mL. Viable counts were determined after 3 h (gray diamonds), 6 h (black squares), and 24 h (white circles). Data are means of three replicate readings. Error bars represent the standard deviation. (b–d) Scanning electron micrographs of EMRSA-16 following growth for 24 h in the presence of (b) DMSO (solvent control), (c) 0.5 μg/mL BBZ8 (inactive compound control), and (d) 0.5 μg/mL BBZ5 (MIC). Scale bars are 3 μm.

Gyrase DNA Binding Inhibition Assays. A 240 bp fragment containing the strong gyrase site (SGS)[30] from pBR322 was amplified by PCR using the specific primers SGS_F (5′-CAA GCC GTC GAC ACT GGT C-3′) and SGS_R (5′-CGC GAG GGA TCC TTG AAG C-3′). The amplified fragment was purified by gel extraction (Qiagen Qiaquick kit) and 3′ end-labeled with DIG-11 ddUTP using terminal transferase according to the manufacturer's instructions (DIG Gel Shift Kit, 2nd Generation, Roche). DNA band shift reaction mixtures (20 μL) contained the indicated amounts of gyrase, DNA, and BBZ compound in the presence of binding buffer [20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.02% Tween (w/v), 10 mM $MgCl_2$, and 30 mM KCl]. The binding reactions were allowed to proceed for 15 min at 37 °C before the mixtures were placed on ice, 2.2 μL of Novex 5x hi-density TBE sample buffer (Invitrogen) was added, and the mixtures were loaded on 6% polyacrylamide gels equilibrated in 0.5× TBEM (TBE and 10 mM $MgCl_2$) at 4 °C. Samples were separated by electrophoresis (70 V for 4 h at 4 °C) before the DNA was transferred onto nylon membranes by electroblotting in 0.5× TBEM (25 V for 2 h). DNA was immobilized on the membrane by being baked at 120 °C for 30 min. Blots were developed using immuno-chemiluminescent detection with the CSPD substrate, as described in the DIG Gel Shift Kit protocol (Roche), and visualized using a GeneGenome gel imager (Syngene). IC_{50} values were determined as described above by densitometry measurement of the bound DNA band.

DNA Gyrase Cleavage Assays. *S. aureus* DNA gyrase (66 nM) was incubated with 500 ng of supercoiled pBR322 in cleavage assay buffer [8 mM HEPES-KOH (pH 7.6), 2 mM magnesium acetate, 2 mM DTT, 0.4 mM ATP, 100 mM potassium glutamate, and 0.01 mg/mL albumin] for 1 h at 37 °C. SDS and proteinase K were added to final concentrations of 0.2% and 0.1 mg/mL, respectively, before a further incubation at 37 °C for 30 min. Reactions were stopped by the addition of 22 μL of 2× STEB and 8 μL of 6× blue-orange loading dye before being loaded directly onto a 1.0% TBE-agarose gel for separation (70 V for 3 h). Gels were visualized, and the nicked circular and linear bands were quantified as described above.

Serial Passage of *S. aureus* in the Presence of BBZ Compounds. MICs were determined in quadruplicate for each compound in 96-well microplates as described above. After incubation for 20 h at 37 °C, the MIC was recorded and cells were recovered from the well containing the highest concentration of BBZ that allowed full growth. These cells were diluted to inoculate a fresh set of plates. This procedure was repeated for 14 days.

RESULTS

Symmetric Bis-benzimidazoles Are Potent Anti-Staphylococcal Agents. A series of BBZ compounds with distinct terminal substitutions were screened for antibacterial activity against clinical and reference strains of *S. aureus* (Table 1). Compounds BBZ4, BBZ5, and BBZ9 exhibited potent antibacterial activity with MIC values between 0.03 and 0.5 μg/mL for both methicillin-susceptible and -resistant strains. This compares very favorably with currently marketed anti-staphylococcal antibiotics, which show MIC values against MRSA of 0.5 μg/mL (vancomycin) to 2 μg/mL (linezolid).[31] The anti-staphylococcal activities of BBZ5 and BBZ9 were also superior to those of other recently reported benzimidazole derivatives, including a structurally similar group of bis-benzimidazole diamidines.[3,4] BBZ6 exhibited moderate activity against the methicillin-sensitive strains but was inactive against EMRSA-16. No other compounds exhibited significant activity against the strains tested. The position and identity of the terminal substituent appeared to be crucial to the activity of the compounds. In particular, the switching of a terminal *p*-methoxy group to the meta position resulted in complete abrogation of activity [compare BBZ9 (MIC = 0.03–0.06 μg/mL) with BBZ8 (MIC > 128 μg/mL)]. To assess the

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mammalian cytotoxicity of the BBZ compounds, we determined IC_{50} values for fibroblast cell line WI-38 using the SRB assay (Table 1). Linear regression analysis of MIC and IC_{50} values gave an r^2 value of ~0.08. This complete lack of correlation suggested either distinct mechanisms of action in prokaryotic and eukaryotic cells or distinct routes for cell entry. An extensive assessment of the antibacterial activities and mammalian cytotoxicities of these compounds will be reported elsewhere (Morreira et al., 2012; P. W. Taylor, manuscript in preparation).

BBZ Compounds Induce Cell Killing and Gross Morphological Defects in *S. aureus*. To determine if the inhibitory activity of BBZ compounds was due to bactericidal or bacteriostatic effects, the viability of three *S. aureus* strains was assessed over time following exposure to increasing concentrations of BBZ9 (Figure 1a). Time-kill kinetics were similar for all *S. aureus* strains tested, revealing a time- and concentration-dependent killing effect similar to that seen for the bactericidal compound ciprofloxacin.[32] Similar results were obtained for BBZ5 (data not shown). The minimal bactericidal concentrations (MBCs) for BBZ9 were 0.1, 1, and 5 μg/mL against NCTC 8325, ATCC 29213, and EMRSA-16, respectively. Compounds can be defined as bacteriostatic if their MBC:MIC ratio is greater than 4.[33] By this definition, BBZ9 is therefore bactericidal against NCTC 8325 and ATCC 29213 but borderline bacteriostatic against EMRSA-16.

The morphological responses of bacteria to antibiotics can provide clues about the mechanism of action and help explain the cell killing properties of bactericidal compounds. To determine if BBZ treatment produced structural abnormalities in EMRSA-16, cells were analyzed by scanning electron microscopy following treatment with growth inhibitory dosages of active compounds BBZ5 and BBZ9 and inactive compound BBZ8 (Figure 1). As negative controls, cells were treated with a volume of DMSO equivalent to the largest BBZ dosage used. DMSO-treated EMRSA-16 appeared as spherical cocci approximately 0.7 μm in diameter (Figure 1b). Similarly, cells exposed to the inactive compound BBZ8 at 1 μg/mL did not exhibit structural defects (Figure 1c). In contrast, treatment with BBZ5 at this concentration resulted in gross morphological abnormalities, including swelling up to 3 times the diameter of control cells, cell lysis, and the appearance of bobblelike protrusions on their cell surface (Figure 1d).

Transcriptional Responses of EMRSA-16 to BBZ5 Treatment. Cell enlargement is a common response to multiple classes of antibiotics, including β-lactams, quinolones, and nitrofurans, and typically occurs either as a result of direct inhibition of the cell division apparatus or as an indirect effect of the SOS response instigated by DNA damage or oxidative stress.[34,35] To determine whether the BBZ compounds instigated either of these responses at the gene level, we identified the transcriptional responses of EMRSA-16 to BBZ challenge using whole genome microarrays. Preliminary experiments performed to determine a suitable dosing regime are described in the Supporting Information. A 15 min treatment with 4 times the MIC of BBZ5 during exponential growth was chosen and had a just-inhibitory effect that was expected to minimize nonspecific responses to the compound (Figure S1 of the Supporting Information). Under these conditions, a total of 198 genes were upregulated and 128 downregulated >2-fold by BBZ5 compared to the DMSO-treated controls (Table S1 of the Supporting Information).

Thirteen of the genes most strongly induced by BBZ5 have roles in DNA replication and repair, detoxification, and oxidative stress responses (Table 2). These included *sodM*, *ahpC*, and *katA*, which have defined or putative roles in the response to oxidative stress, as well as *recA*, which is induced following DNA damage as the initial component of the bacterial SOS response.[35,36] In addition, all four genes encoding DNA gyrase and topo IV were significantly upregulated by BBZ5. Other BBZ5-regulated genes could broadly be grouped into three categories: (i) genes encoding transport proteins, including Na^+/H^+ antiporters, oligopeptide ABC transporters, and amino acid transporters; (ii) genes regulating small molecule metabolism, including purine and fatty acid biosynthesis; and (iii) genes involved with cell envelope biosynthesis through production of peptidoglycan, phospholipids, and the Gram-positive capsule. These responses were consistent with an overall strategy of the bacteria to reduce the intracellular concentration of the compound, prevent its further entry, and repair and protect damaged DNA.

Table 2. Genes Controlling DNA Replication and Repair, Detoxification, and Oxidative Stress Responses That Were Significantly Upregulated in EMRSA-16 and NCTC 8325 following 15 min Exposures to BBZ5 at Concentrations Equivalent to 4 Times the MIC

	relative level of expression[a]			
gene	EMRSA-16	NCTC 8325	gene product	product function
sodM	7.48	5.07	superoxide dismutase	antioxidant enzyme
recF	3.29	2.20	DNA replication and repair protein RecF	repair of single-stranded DNA breaks/SOS response
recA	3.31	2.76	recombinase A	homologous recombination/SOS response
nusG	3.08	2.60	transcription antitermination protein	regulation of transcription
katA	2.58	0.82[b]	catalase	peroxide protection
gyrB	2.86	2.23[b]	DNA gyrase subunit B	DNA gyrase ATPase domain
gyrA	2.64	2.46	DNA gyrase subunit A	DNA gyrase DNA binding domain
groES	2.69	5.44	GroES protein	chaperonin/heat-shock protein
groEL	2.65	4.49	GroEL protein	chaperonin/heat-shock protein
grlB	3.25	2.90	topoisomerase IV subunit B	topoisomerase IV ATPase domain
grlA	2.99	2.86	topoisomerase IV subunit A	topoisomerase IV DNA binding domain
dnaG	2.34	2.17	DNA primase	DNA synthesis enyme
ahpC	2.85	3.34	alkyl hydroperoxide reductase subunit C	peroxide resistance

[a]Relative to DMSO-treated control ($P < 0.05$). [b]These values were not statistically significant in this strain ($P < 0.05$).

BBZ Compounds Inhibit *S. aureus* DNA Gyrase Supercoiling Activity. Exposure to gyrase inhibitors such as novobiocin, ciprofloxacin, norfloxacin, and simocyclinone D8 (SD8) increases the level of expression of *gyrA* and *gyrB* in Gram-positive and Gram-negative bacteria.[37−40] The microarray data were therefore consistent with BBZ-mediated inhibition of bacterial topoisomerases, leading to DNA damage and oxidative stress. Such an effect could account for both the

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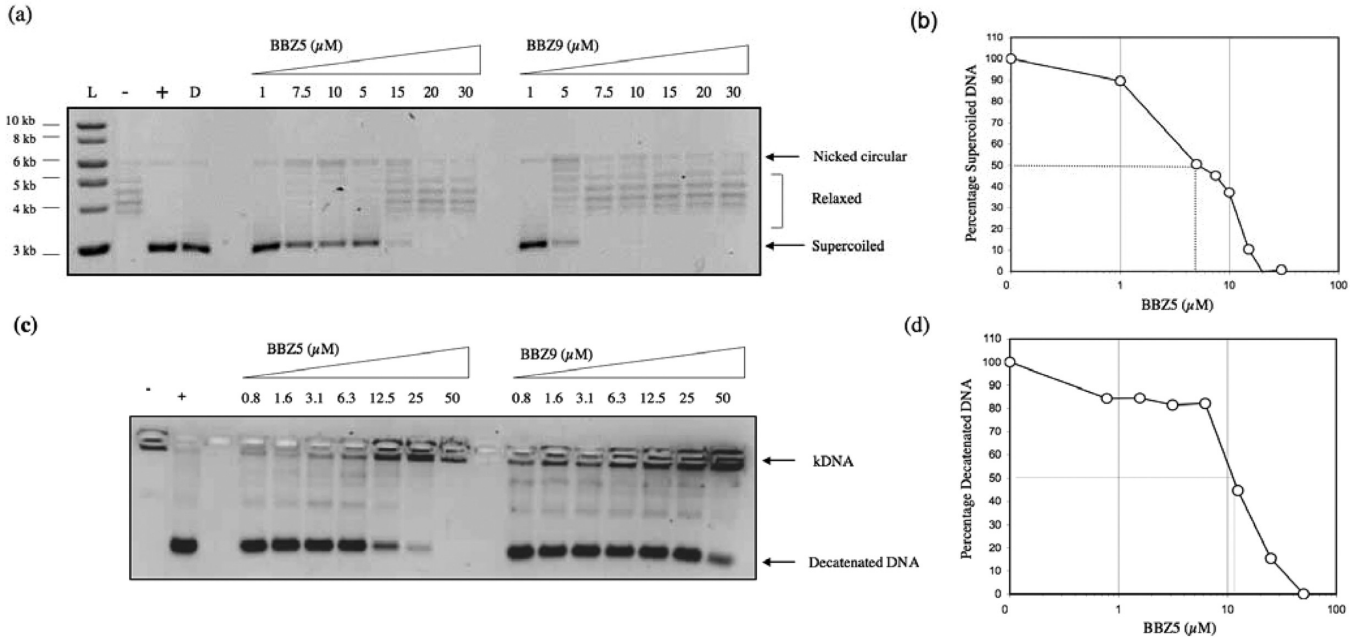

Figure 2. BBZ compounds inhibit the physiological functions of *S. aureus* type II topoisomerases. (a) Inhibition of DNA gyrase-mediated supercoiling. Supercoiled pBR322 was incubated with *S. aureus* DNA gyrase with the indicated concentrations of test compounds: lane L, 2 log DNA ladder; lane −, relaxed pBR322 alone; lane +, DNA gyrase no drug control; lane D, DNA gyrase with a 2.5% DMSO control. (c) Inhibition of *S. aureus* topo IV decatenation activity. Kinetoplast DNA (kDNA) was incubated with *S. aureus* topo IV and increasing concentrations of test compounds as indicated. (b and d) Representative graphs for the determination of IC_{50} values for DNA supercoiling and decatenation inhibition, respectively. The intensity of the supercoiled or decatenated bands for each drug concentration was plotted as a percentage of the intensity of that band for the positive control. IC_{50} values were taken by interpolation from 50% supercoiled or decatenated DNA as indicated by dashed lines.

bactericidal effects of these compounds in *S. aureus* and the observed morphological responses. To test this theory, the ability of the BBZ compounds to inhibit the primary physiological functions of *S. aureus* DNA gyrase (supercoiling) and topo IV (decatenation) was assessed using in vitro biochemical assays (Figure 2). To confirm the sensitivity of these enzymes to established inhibitors, and for comparison, IC_{50} values for ciprofloxacin and novobiocin were also determined.

Compounds were initially tested at 50 μM. IC_{50} values were then determined for any compounds that exhibited inhibitory activity at this concentration by measurement of the intensity of supercoiled or decatenated bands (Table 3). In agreement with previously shown specificities for the reference compounds in *S. aureus*, novobiocin strongly inhibited DNA gyrase but had little activity against topo IV, whereas the opposite was observed for ciprofloxacin.[41] Topo IV-catalyzed decatenation was moderately inhibited by BBZ5. The remaining BBZ compounds displayed no significant inhibitory activity against this enzyme at concentrations below 50 μM. In contrast, of the 10 BBZ compounds tested, those showing potent antibacterial activity, BBZ5, BBZ4, and BBZ9, inhibited DNA gyrase-catalyzed DNA supercoiling with IC_{50} values of ∼5−10 μM. These potencies were comparable to those previously reported for novobiocin and superior to those reported for ciprofloxacin in *S. aureus*.[41] These data therefore strongly support the hypothesis that DNA gyrase is the BBZ molecular target. Surprisingly, however, BBZ8, which displayed no antibacterial activity, also inhibited DNA gyrase, with an IC_{50} value of 8.8 μM.

Table 3. Inhibitory Activities of BBZ Compounds and Reference Antibiotics against *S. aureus* DNA Gyrase-Mediated Supercoiling and Topo IV-Mediated Decatenation Activities

	IC_{50} (μM)[a]	
Compound	DNA gyrase supercoiling	topo IV decatenation
BBZ4	4.74 ± 1.42	53.0 ± 30.87
BBZ5	10.29 ± 3.28	15.45 ± 3.36
BBZ8	8.82 ± 4.70	70.33 ± 15.31
BBZ9	5.58 ± 1.57	50.87 ± 4.70
novobiocin	1.55 ± 0.21	27.5
ciprofloxacin	>50	8.28 ± 1.71

[a]Data given are means ± the standard deviation of at least three biological replicates where indicated.

The Transcriptional Response to BBZ Treatment Is Distinct from That of Ciprofloxacin and Novobiocin. To establish if the BBZ compounds triggered transcriptional responses similar to those produced by known topoisomerase inhibitors, we measured mRNA transcript abundances in *S. aureus* cells exposed to BBZ5, BBZ8, BBZ9, novobiocin, and ciprofloxacin. *S. aureus* NCTC 8325 was used in these experiments as it is a sequenced and well-established reference strain, which harbors no antibiotic resistance determinants that could mask the expected antibiotic response. To minimize general stress responses, subinhibitory dosages of each compound were determined on the basis of previously published guidelines for antibacterial transcriptional profiling.[42] *S. aureus* NCTC 8325 was then challenged for 15 min at midlog phase with doses of each compound that gave comparable growth inhibitory responses (Figure S2 of the Supporting Information). Transcriptional response profiles for the five treatments are summarized in Figure 3. Both BBZ5 and BBZ9 treatments resulted in pronounced and highly similar expression profiles that clustered separately from BBZ8,

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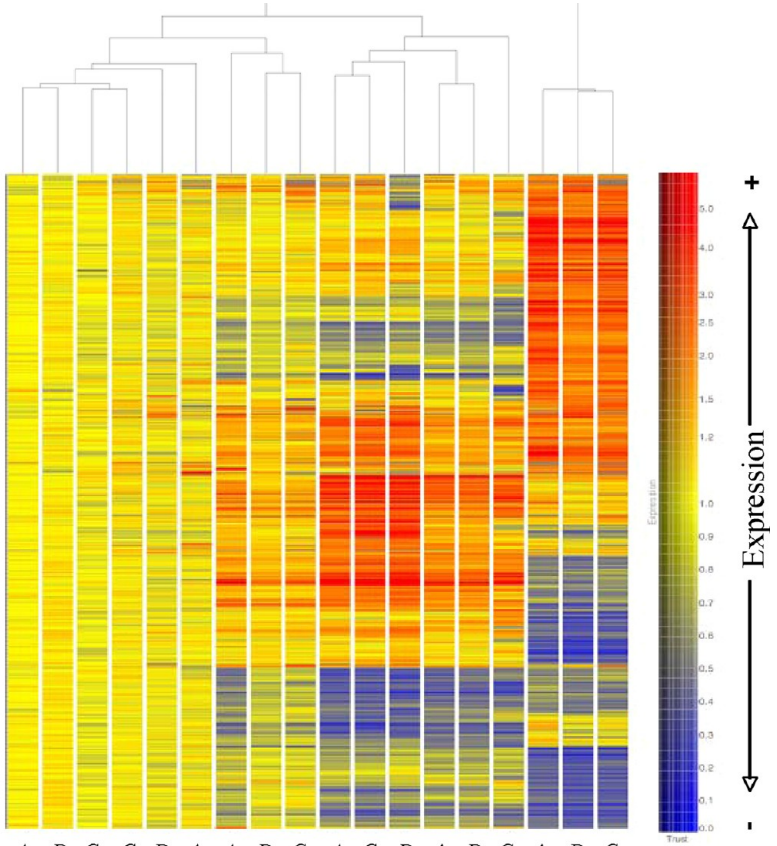

Number of genes significantly:	Up-regulated	1	22	231	108	345
	Down-regulated	0	4	130	98	220

Figure 3. Transcriptional responses of *S. aureus* NCTC 8325 to treatment with symmetric bis-benzimidazoles and two characterized topoisomerase inhibitors. Hierarchical clustering displaying transcriptional response profiles following 15 min exposures to subinhibitory doses of ciprofloxacin (CIP), BBZ8, BBZ5, BBZ9, and novobiocin (NOV). Each column represents a single biological replicate and treatment. Replicates were clustered in a manner that was independent of their annotation, by Spearman correlation of their gene expression levels. Each horizontal bar represents one gene. Genes are colored by their expression level. Genes for which expression was upregulated relative to the control are colored red, and downregulated genes are colored blue. Yellow indicates no change in expression relative to the control. The table lists the total numbers of genes up- and downregulated >2-fold by each treatment compared to the control ($P < 0.05$ via ANOVA using Benjamini and Hochberg correction for false discovery rate correction).

novobiocin, and ciprofloxacin (Figure 3). Hundreds of genes were up- or downregulated by each treatment. The transcriptional response of *S. aureus* NCTC 8325 to novobiocin was similar to that previously observed in *Haemophilus influenzae*[37] but was visibly distinct from that of BBZ5 and BBZ9, strongly indicating different modes of action. Despite causing growth inhibition at visibly similar levels compared with the other treatments (Figure S2 of the Supporting Information), the only gene whose expression was significantly affected in ciprofloxacin-treated cells was *recA*. This suggested that the kinetics of the response to BBZ and ciprofloxacin were distinct. All but two of the DNA damage and repair-related genes that were significantly upregulated by BBZ5 in EMRSA-16 were also upregulated by BBZ5 and BBZ9 in *S. aureus* NCTC 8325 (Table 2 and Table S2 of the Supporting Information). In contrast, treatment with the inactive compound BBZ8 did not significantly affect the expression of *gyrAB*, *grlAB*, *recA*, or *recF*. A full description and discussion of the transcriptional responses to BBZ treatment is given in the Supporting Information.

The results of the microarray experiments were independently confirmed by qRTPCR. Genes belonging to different functional categories that were upregulated (*gyrA*, *grlA*, *purC*, and *sarR*), downregulated (*fabH* and *mnhD*), and unchanged (*cmk*) by BBZ treatment were assessed for their extent of regulation in each of the five NCTC 8325 drug treatments relative to the DMSO-treated control (Table S3 of the Supporting Information). Of 33 conditions tested, 30 values for fold regulation by qRTPCR agreed qualitatively with the values obtained by microarray, and of these, 20 qRTPCR fold changes, including those for topoisomerase genes *gyrA* and *grlA*, were <2-fold different from the microarray values (Figure S3 of the Supporting Information). Linear regression analysis of this data gave an r^2 value of 0.67, indicating a good correlation between the gene regulation values obtained by the two techniques and largely confirming the results of the microarray experiments.

Overall, the transcriptional profiling experiments further confirmed the relevance of type II topoisomerases and DNA damage responses to BBZ-induced stress and also suggested

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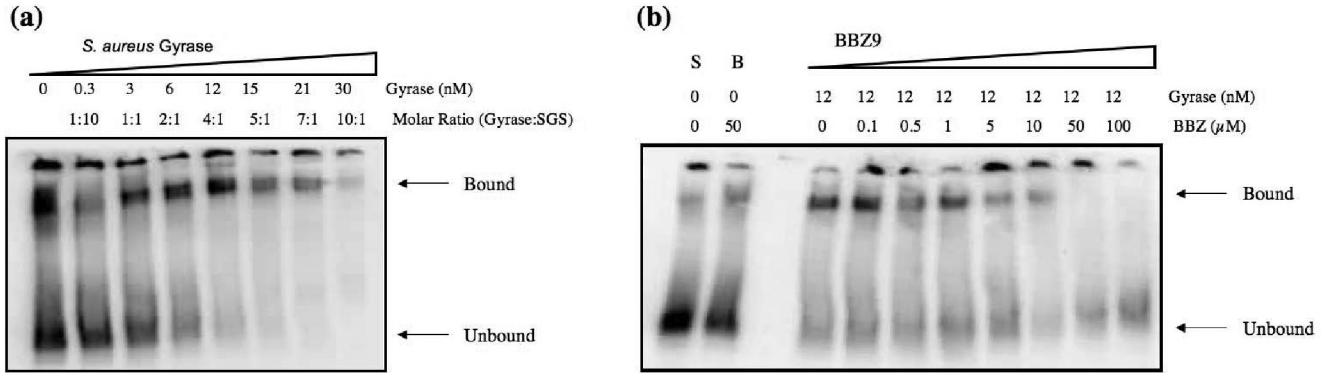

Figure 4. BBZ9 prevents DNA gyrase from binding to DNA. Shown are DNA band shift assays performed to assess the binding of *S. aureus* DNA gyrase to a 240 bp DNA fragment containing the SGS. Assays were performed as described in Experimental Procedures. Each lane contained 3 nM DIG-labeled SGS and the indicated concentrations of *S. aureus* DNA gyrase and/or BBZ compounds. (a) Optimization of the gyrase−DNA binding reaction for maximal sensitivity. SGS:DNA gyrase molar ratios are given. (b) Binding of gyrase to DNA is inhibited by increasing concentrations of BBZ9. Arrows indicate bands that correspond to unbound and gyrase-bound DNA.

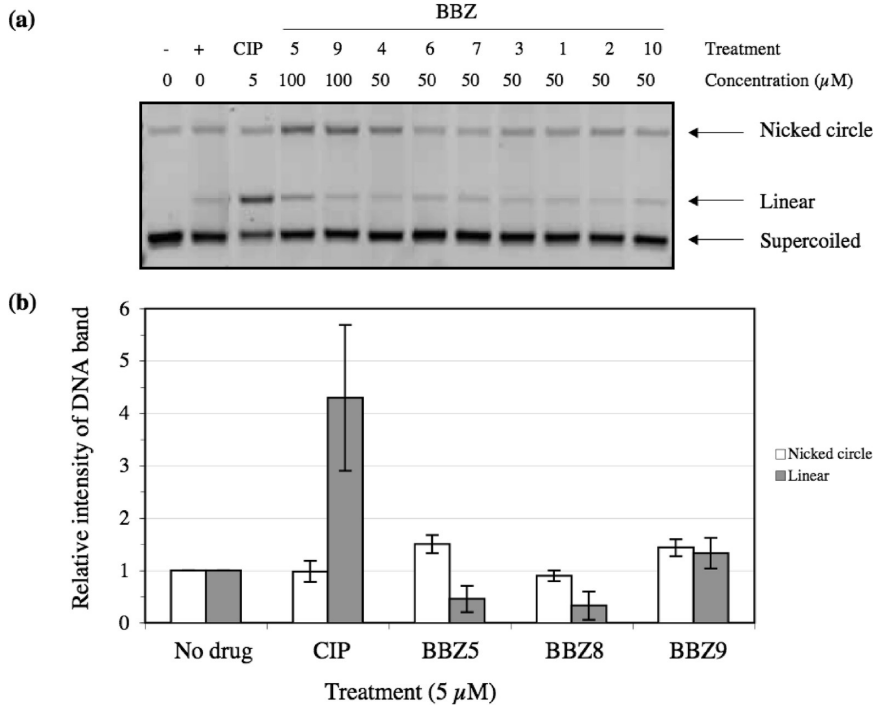

Figure 5. Incubation of *S. aureus* DNA gyrase with BBZ compounds results in the accumulation of nicked circular DNA. (a) Shown is an initial screening assay for DNA cleavage activity. Supercoiled pBR322 was incubated with *S. aureus* DNA gyrase and the indicated concentrations of ciprofloxacin (CIP) or BBZ compounds, as described in Experimental Procedures. (b) Quantification of nicked circular (white bars) and linear (gray bars) DNA bands. The relative cleavage activities of DNA gyrase in the presence of 5 μM CIP, BBZ5, BBZ8, and BBZ9 were determined by measurement of the intensities of linear and nicked circular bands relative to the no drug control, for which band intensities were normalized to 1. Data are means of three to five independent replicate assays. Error bars indicate the standard deviation.

that the mechanism of gyrase inhibition was distinct from that of the quinolones and the coumarins.

BBZ Compounds Have Dual Inhibitory Activities against *S. aureus* DNA Gyrase. The ability of BBZ8 to inhibit DNA gyrase suggested that the functional inhibition of type II topoisomerases might not be the driving factor for the antibacterial effects of the BBZ compounds. To determine if this discrepancy was linked to the molecular mechanism of gyrase inhibition, we performed two further experiments. The DNA gyrase inhibitor SD8 prevents the binding of the enzyme to DNA, thus inhibiting DNA gyrase-mediated supercoiling and relaxation.[14,43] To determine if the BBZ compounds shared this inhibitory mechanism, the binding of DNA gyrase to a DNA substrate containing the SGS gyrase binding site was assessed using band shift assays. The mobility of a labeled 240 bp DNA fragment containing the SGS was reduced by the addition of *S. aureus* DNA gyrase, demonstrating binding of the enzyme to the DNA (Figure 4a). A 4:1 gyrase:SGS molar ratio was the minimum required to shift the majority of the labeled DNA. Using this molar ratio to maximize sensitivity, band shift reactions were then repeated with the addition of increasing concentrations of BBZ9 (Figure 4b). At 5−10 μM BBZ9, a shift from the bound to the unbound state became noticeable, and at 50−100 μM BBZ9, binding of gyrase to the DNA was

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completely abolished. By measurement of the band corresponding to gyrase-bound DNA, the IC_{50} value for this activity for BBZ9 was ~3 μM. Addition of BBZ9 alone did not alter the mobility of the DNA. Similar results were obtained for the uncharged compounds BBZ4 and BBZ8 (data not shown). However, addition of BBZ5 to a final concentration of 100 μM failed to elicit a band shift. This result could be an artifact resulting from the positive charge of BBZ5 that could prevent its interaction with the DNA gyrase–DNA complex during PAGE separation; it could also reflect differing modes of topoisomerase inhibition for BBZ5 compared with BBZ9. From these results, we concluded that uncharged BBZ compounds prevent the binding of DNA gyrase to DNA, contributing to or accounting for their anti-gyrase activities, but that this binding inhibition does not fully account for the antibacterial nature of these compounds.

BBZ5 and BBZ9 exposure induced expression of the DNA damage response genes *recA* and *recF*, indicating that the compounds caused accumulation of double-stranded or single-stranded DNA breaks (Table 2 and Table S2 of the Supporting Information). To determine if BBZ compounds stabilized the gyrase–DNA cleavage complex in a manner similar to that of the quinolones, ciprofloxacin or BBZ compounds were incubated at varying concentrations with DNA gyrase and supercoiled DNA in the presence of ATP. Linear DNA intermediates were separated from the covalent complexes by the addition of SDS and proteinase K (Figure 5a). Treatment of the supercoiled DNA with gyrase alone resulted in the appearance of a weak linear band, reflecting the equilibrium of the reaction. The addition of ciprofloxacin at 5 μM resulted in a >4-fold increase in the intensity of the linear band relative to that of untreated gyrase, demonstrating the expected accumulation of cleaved DNA intermediates. In contrast, the addition of BBZ compounds to a final concentration of 100 μM did not significantly increase the abundance of the linear band but instead decreased the abundance of the linear DNA product relative to the gyrase positive control. It was noted that addition of the BBZ compounds that displayed antibacterial activity led to a visible accumulation of the nicked circular band corresponding to DNA harboring single-stranded breaks. The accumulation of nicked DNA is consistent with the trapping of a covalent, single-stranded cleavage complex and is a known cytotoxic lesion produced by bacterial topoisomerase I inhibitors and by the quiniline derivative class of anti-staphylococcal topoisomerase inhibitors.[15,44,45] To confirm these observations, we repeated the cleavage assays four more times with ciprofloxacin, BBZ5, BBZ8, and BBZ9 at concentrations of 5 μM. The intensities of the nicked circular and linear bands were quantified for each gel and expressed as a proportion of the band intensity observed for the gyrase only control (Figure 5b). The accumulation of nicked circular DNA was consistently observed following treatment with antibacterial BBZ compounds BBZ5 and BBZ9; BBZ8 produced no significant accumulation of this intermediate at 5 μM. This result therefore provided strong evidence that BBZ-mediated *S. aureus* cell killing was a consequence of gyrase inhibition leading to the accumulation of single-stranded DNA breaks.

Resistance to BBZ Compounds Does Not Readily Emerge in *S. aureus*. The final proof for mode of action studies is often the demonstration of a link between drug resistance and a genetic alteration in the proposed target. Resistance to bacterial topoisomerase inhibitors arises readily through point mutations that prevent the interactions between the drug and enzyme.[46–48] In an attempt to confirm gyrase as the molecular target of the BBZ compounds in *S. aureus*, we conducted a serial passage experiment to isolate resistant strains. *S. aureus* NCTC 8325 and EMRSA-16 were grown in the presence of BBZ5 and BBZ9 between 0.03 and 16 μg/mL for 14 days (Figure 6). No significant increase in MIC was observed over this period, indicating that single-point mutations that would be expected to accumulate were insufficient to confer resistance to the compounds. It was therefore not possible to confirm genetically that gyrase inhibition is the sole or driving mechanism of BBZ-mediated cell killing in *S. aureus*.

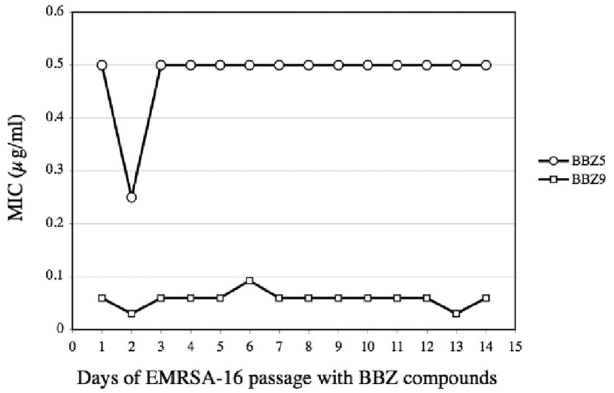

Figure 6. BBZ-resistant *S. aureus* did not arise following a 14 day serial passage. MIC values for EMRSA-16 grown in the presence of increasing 2-fold dilutions (16–0.03 μg/mL) of BBZ5 (○) and BBZ9 (□) were determined for 14 consecutive days as described in Experimental Procedures. Similar results were obtained for NCTC 8325.

DISCUSSION

The bacterial topoisomerases are established antibacterial targets for which novel inhibitors are still being actively sought. Their suitability as drug targets is linked with their essential nature, the bactericidal consequences of their inhibition, and the presence of multiple drug interaction sites that are exploited by numerous antibacterial agents.[9,49] In addition, DNA gyrase and topo IV are sufficiently structurally similar to accommodate dual inhibition, reducing rates of spontaneous resistance for compounds that are able to inhibit both enzymes simultaneously.[50] One common group of topoisomerase inhibitors, the coumarins such as novobiocin, has limited clinical utility because of the toxicity and poor drug characteristics of its members.[11,51] In addition, resistance to these compounds readily arises.[52] In contrast, the quinolones such as ciprofloxacin are commonly used in the clinic.[53] However, resistance to the quinolones is also widespread. In a study of 34 MRSA isolates, all but one appeared to be resistant to currently marketed drugs, including ciprofloxacin and clinafloxacin.[54] New compounds with novel mechanisms of action against these enzymes are therefore particularly desirable for overcoming existing pools of resistant organisms and circumventing resistance barriers to clinical utility. The data presented here indicate that the BBZ compounds may meet these demands, making them potentially useful clinical agents. Of note, the mechanism of BBZ-mediated topoisomerase inhibition is complicated and multifaceted, and resistance against the compounds does not appear to readily occur, which may be

ultimately significant should these or related compounds eventually enter the clinic.

DNA binding and cleavage assays for *S. aureus* DNA gyrase gave positive indications for two mechanisms of BBZ-mediated gyrase inhibition that are shared by two novel classes of topoisomerase inhibitors. We have shown using an electrophoretic mobility shift assay that the uncharged compounds BBZ4, BBZ8, and BBZ9 can interfere with the binding of DNA gyrase to DNA. This inhibitory mechanism has recently been described for SD8, a bifunctional molecule consisting of polyketide and aminocoumarin moieties that interact with two separate binding pockets in the N-terminal domain of GyrA, in a region adjacent to the quinolone resistance-determining region (QRDR).[14,43] This activity results in transcriptional profiles in *Escherchia coli* that are related to, but distinct from, those of BBZ5, novobiocin, and the quinolone norfloxacin.[40] In contrast with BBZ4, BBZ8, and BBZ9, the charged compound BBZ5 surprisingly did not interfere with DNA binding. In addition, BBZ5 moderately inhibited the decatenation activity of topo IV. SD8 has no appreciable activity against topo IV, indicating that BBZ5 acts in a manner distinct from that of this compound.

All three BBZ compounds that exhibited antibacterial activity caused the accumulation of nicked circular DNA corresponding to single-stranded breaks in DNA cleavage assays. The generation of these DNA lesions provides a clear explanation for both the DNA damage responses identified in the transcriptional analysis that included upregulation of *recA* and *recF*, the bactericidal effects identified by time-kill curves, and the observed cell swelling and lysis. The quiniline derivative class of compounds that share this mechanism of action has been shown to do so by inhibiting DNA gyrase prior to double-stranded cleavage, thus stabilizing an equilibrium state that contains both uncleaved DNA and single-stranded nicks.[15] These compounds are ~1000 times more potent as inhibitors of DNA gyrase than both ciprofloxacin and the BBZ compounds, with IC_{50} values of 14 ± 5 nM for *S. aureus* DNA supercoiling inhibition and MIC values of ≤0.016 μg/mL against MRSA. The superior potency of these compounds compared with the potency of the BBZ series may indicate that the two classes of compounds cause accumulation of single-stranded breaks by different mechanisms.

A 2 week serial passage in the presence of BBZ5 and BBZ9 failed to yield any increase in MIC values. In contrast, resistance to both established and novel topoisomerase inhibitors is readily generated in the laboratory. These mutations are used to define the sites of drug−enzyme interaction. For example, mutations conferring resistance to the quiniline derivatives are located in a region proximal to but distinct from the QRDR.[15] Other benzimidazole derivatives with anti-MRSA activity have previously been shown to bind to the minor groove of bacterial DNA at A/T rich target sequences commonly found in bacterial promoters and replication origins.[55] Similarly, cationic BBZ derivatives have previously been shown by both X-ray crystallography and DNA footprinting to bind duplex DNA at runs of four consecutive AT residues, in a sequence selective manner.[16,17] Taking this into account, we propose the following as plausible models of BBZ-mediated DNA gyrase inhibition. First, initial weak interactions occur between BBZ and dsDNA that in themselves have no antibacterial consequences. Once DNA gyrase and/or topo IV becomes involved, the interactions are stabilized by formation of a ternary complex. Alternatively, cooperative weak binding interactions with DNA in AT rich stretches may result in localized alterations in the tertiary structure of the bacterial chromosome, thus both preventing the binding of DNA by topoisomerases and interfering with the correct functioning of topoisomerases and other multiprotein machineries already attached to the DNA. Both of these models could account for the pleiotropic inhibitory mechanisms observed against DNA gyrase: enzymes not yet attached to duplex DNA could be prevented from doing so, while those already attached could be stabilized or disrupted at an intermediate stage of DNA cleavage religation, resulting in single-stranded DNA breaks.

Difficulty in generating BBZ resistance in vitro indicated that single-point mutations are insufficient for conferring drug resistance, and these models could therefore also explain this phenomenon. Another possible explanation for the low rate of resistance is that there are multiple targets for the BBZ compounds. The topoisomerase inhibitor VRT-125853 shows a low frequency of spontaneous resistance due to balanced inhibition of both DNA gyrase and topo IV.[5] BBZ5 also inhibits both enzymes with similar IC_{50} values, which may explain the lack of resistance to this compound. Furthermore, we do not rule out the possibility that the BBZ compounds may possess additional inhibitory activities that have not been identified in this study.

As the need to confront the emergence of antibacterial resistance becomes more urgent, the identification of antibacterial compounds that attack existing validated targets, at new sites and through new mechanisms of action, is becoming an important strategy in the development of new therapies.[56] Here we have described the potent antibacterial activity of a series of symmetric bis-benzimidazoles against a clinically relevant strain of MRSA. These compounds inhibit the physiological roles of *S. aureus* topoisomerases by both preventing these enzymes from binding to DNA and stabilizing a single-stranded cleavage complex, leading to the accumulation of lethal DNA lesions. In an earlier study, similar BBZ derivatives were shown to weakly inhibit human topoisomerase IIα-mediated decatenation and DNA relaxation.[17] The micromolar cytotoxicity in a human fibroblast cell line of six of the BBZ compounds presented here (Table 1) may be linked with this activity, and these interactions will need to be thoroughly assessed in the future as sources of potential toxicity if the compounds are to proceed to clinical utility. However, for BBZ4, BBZ5, and BBZ9, mammalian cytotoxicity values were between 8- and 60-fold lower than their corresponding MICs, indicating a strong preference for bacterial topoisomerases. Furthermore, in contrast with *S. aureus* DNA gyrase, inhibition of human topoisomerase IIα with BBZ compounds was not accompanied by accumulation of single- or double-stranded DNA breaks, which is likely to be the key cytotoxic event in bacteria. The compounds also exhibited lower mammalian cytotoxicity than novobiocin or ciprofloxacin (the latter is still in clinical use). Taken together, these observations indicate that the mechanisms of bacterial and human gyrase inhibition are distinct and that there is a potential therapeutic window for these compounds as antibacterials.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Growth responses of *S. aureus* to the treatments used for microarray analysis, detailed transcriptional response data, and qRTPCR validation data for the microarray analysis. This

material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All experimental work was performed by A.G.D. Microarray design and assistance with microarray data analysis were provided by J.H. The BBZ compounds were synthesized by J.M. P.W.T. provided assistance with microbiological assessments. The manuscript was written by A.G.D. and S.N.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BBZ, symmetric bis-benzimidazole; MRSA, methicillin-resistant *S. aureus*; MIC, minimal inhibitory concentration; SRB, sulforhodamine B; SGS, strong gyrase site; MBC, minimal bactericidal concentration; qRTPCR, quantitative real-time polymerase chain reaction; topo IV, topoisomerase; SD8, simocyclinone D8; QRDR, quinolone resistance-determining region; CIP, ciprofloxacin; Nov, novobiocin.

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