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Development and Validation of a High-Throughput Cell-Based Screen To Identify Activators of a Bacterial Two-Component Signal Transduction System

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CpxRA is a two-component signal transduction system (2CSTS) found in many drug-resistant Gram-negative bacteria. In response to periplasmic stress, CpxA autophosphorylates and donates a phosphoryl group to its cognate response regulator, CpxR. Phosphorylated CpxR (CpxR-P) upregulates genes involved in membrane repair and downregulates multiple genes that encode virulence factors, which are trafficked across the cell membrane. Mutants that constitutively activate CpxRA in *Salmonella enterica* serovar Typhimurium and *Haemophilus ducreyi* are avirulent in mice and humans, respectively. Thus, the activation of CpxRA has high potential as a novel antimicrobial/antivirulence strategy. Using a series of *Escherichia coli* strains containing a CpxR-P-responsive *lacZ* reporter and deletions in genes encoding CpxRA system components, we developed and validated a novel cell-based high-throughput screen (HTS) for CpxRA activators. A screen of 36,000 compounds yielded one hit compound that increased reporter activity in wild-type cells. This is the first report of a compound that activates, rather than inhibits, a 2CSTS. The activity profile of the compound against CpxRA pathway mutants in the presence of glucose suggested that the compound inhibits CpxA phosphatase activity. We confirmed that the compound induced the accumulation of CpxR-P in treated cells. Although the hit compound contained a nitro group, a derivative lacking this group retained activity in serum and had lower cytotoxicity than that of the initial hit. This HTS is amenable for the screening of larger libraries to find compounds that activate CpxRA by other mechanisms, and it could be adapted to find activators of other two-component systems.

The increasing prevalence of multidrug-resistant Gram-negative bacteria has prompted urgent calls for new antibiotics (1). *Escherichia coli* sequence type 131, a clonal group that expresses extended-spectrum β-lactamases (ESBLs) and quinolone resistance, has emerged as a major cause of community- and health care-associated urinary tract infections in the United States (2–4). The *Klebsiella pneumoniae* carbapenemase (KPC) has rendered some strains of *K. pneumoniae* resistant to all β-lactams, while the New Delhi metallo- (NDM-1) β-lactamase-containing plasmid has rendered some strains of *E. coli* and *K. pneumoniae* panresistant (5–9). These developments raise the specter that several common infections, such as urinary tract infections due to *E. coli* or *K. pneumoniae*, may soon be caused by organisms that are virtually untreatable (5, 8, 9).

The traditional approach to discover antibiotics has been to screen libraries of natural or synthetic products for bacterial killing activity in culture. Unfortunately, this strategy has yielded no new targets or classes of drugs for Gram-negative bacteria over the past 50 years (10–12). More contemporary approaches are aimed at identifying inhibitors of novel targets essential for growth or virulence. Attractive targets include bacterial two-component signal transduction systems (2CSTS), which typically consist of a sensor kinase (SK) and a response regulator (RR), have no mammalian homologs, and involve the phosphorylation of amino acids that differ from the targets of mammalian phosphatases and kinases. Although several inhibitors of 2CSTS have antibacterial activity *in vitro* (13–16), none have achieved clinical utility in humans. The failure to develop inhibitors may be due to the redundancy of 2CSTS or to the poor selectivity and bioavailability of these compounds, which target the hydrophobic active site of the SK (15, 16). Another approach has been to find nontraditional therapeutics that target 2CSTS and do not cause cell death but downregulate the expression of virulence factors (13, 16, 17). For example, inhibition of the 2CSTS QseBC by the small molecule LED209 increased survival in animals infected with *Salmonella enterica* serovar Typhimurium or *Francisella tularensis* (18, 19). Thus, there is a rationale to expand the repertoire of nontraditional therapeutics that target 2CSTS.

CpxRA is a 2CSTS that allows Gram-negative bacteria to sense

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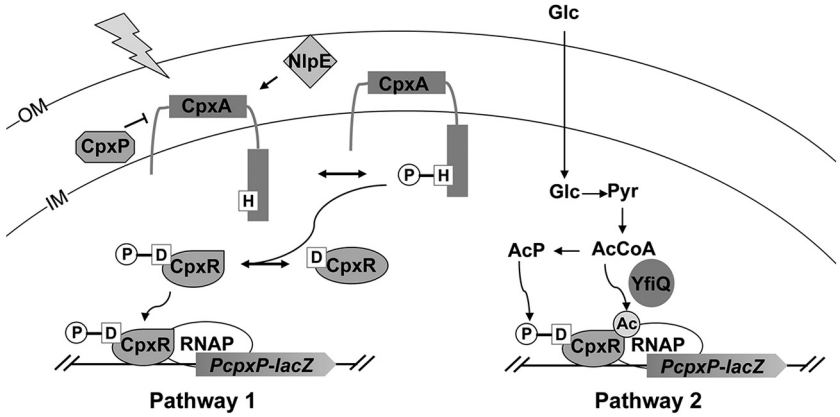


FIG 1 CpxRA two-component signal transduction system (27). In response to membrane stress, CpxP dissociates from CpxA. CpxA phosphorylates on a conserved histidine residue and donates a phosphate group to a conserved aspartic acid residue on CpxR (pathway 1, left). A *PcpxP-lacZ* transcriptional fusion serves as a reporter for CpxR activity. In response to glucose, CpxR can accept phosphoryl groups from acetyl phosphate (AcP), and transcription requires the acetylation of RNA polymerase (RNAP) and YfiQ (pathway 2, right) (29). Compounds that activate CpxRA might target CpxP, NlpE, CpxA, CpxR, or YfiQ (29).

and respond to envelope stress (20–23). CpxA is an SK that spans the cytoplasmic membrane, and CpxR is its cognate RR. Upon sensing membrane stress, CpxA autophosphorylates on a conserved histidine residue and subsequently donates a phosphate group to a conserved aspartic acid residue on CpxR (20) (Fig. 1). In *E. coli*, to alleviate membrane stress, phosphorylated CpxR (CpxR-P) regulates the transcription of approximately 100 genes; genes that maintain envelope integrity are upregulated, whereas genes that encode secreted factors are downregulated (24–26). In *E. coli*, CpxA activity is regulated by two upstream components, CpxP and NlpE (27) (Fig. 1). The periplasmic chaperone CpxP inhibits CpxA kinase activity. Misfolded proteins bind to CpxP and cause it to dissociate from CpxA, activating the system. By some unknown mechanism, surface adhesion induces the lipoprotein NlpE to activate CpxA.

In addition to being an SK, CpxA also has phosphatase activity for CpxR-P (20). In the absence of envelope stress, CpxA acts as a net phosphatase, and CpxR remains inactive. When wild-type cells are grown in minimal medium containing excess carbon, such as 0.4% glucose, CpxR is activated by accepting phosphoryl groups from small-molecule donors, such as acetylphosphate (AcP) (28) (Fig. 1). Glucose-induced activation requires the lysine acetyltransferase YfiQ (also known as Pka and PatZ) and the acetylation of lysine 298 of the RNA polymerase α-subunit but does not require CpxA (28–30). A *cpxA* deletion mutant (Δ*cpxA*) responds more robustly to glucose than does its wild-type parent, because the Δ*cpxA* mutant lacks phosphatase activity and accumulates CpxR-P (28, 30). *cpxA** alleles, which carry mutations in the region encoding the sensing domain, result in constitutive phosphorylation of CpxR; such mutants accumulate even higher levels of CpxR-P than do Δ*cpxA* mutants (21, 28).

CpxRA is found in many drug-resistant bacteria, including *Haemophilus ducreyi*, *Neisseria gonorrhoeae*, *E. coli*, and *K. pneumoniae*, and is highly conserved across the *Enterobacteriaceae* (21, 31). In several of these pathogens, activation of CpxR by the deletion of *cpxA* or by *cpxA** mutations reduces the expression of virulence determinants, consistent with the fact that a major function of the system is to reduce protein flow to the periplasm. An *H. ducreyi* Δ*cpxA* mutant reduces the expression of seven virulence determinants that are required for human infection (32–34). When inoculated into the skin of human volunteers, the *H. ducreyi* Δ*cpxA* mutant is avirulent (32). In contrast, an *H. ducreyi* Δ*cpxR* mutant, which maintains wild-type levels of virulence determinant expression, is fully virulent in humans (33, 35). Similarly, constitutive activation of CpxRA abolishes *S. enterica* serovar Typhimurium virulence in mice (36). Mice fed a lethal dose of the wild type and Δ*cpxR* mutant become infected, while those fed similar doses of the Δ*cpxA* and *cpxA** mutants do not (36). Furthermore, Δ*cpxA* mutants of uropathogenic *E. coli* (UPEC) and *N. gonorrhoeae* are outcompeted by the wild type by three orders of magnitude in their respective murine infection models (A. Jerse and S. Spinola, unpublished data, and H. Mobley and S. Spinola, unpublished data). Taken together, these data led us to hypothesize that activating CpxRA may be a broadly applicable antivirulence strategy and that compounds that pharmacologically activate CpxRA will downregulate virulence determinants and allow the host immune response to clear the infection.

To begin testing this hypothesis, we developed a high-throughput screen (HTS) to detect compounds that activate CpxRA using an *E. coli* strain containing a CpxR-responsive *lacZ* reporter (27). To identify the components required for compound-induced activation, we used an isogenic set of *cpxRA* mutants. Finally, we present validation of the screen and the strategy used to characterize the targets of hit compounds. The screen should be amenable for the discovery of compound leads with the potential to cripple the virulence of multidrug-resistant Gram-negative pathogens.

MATERIALS AND METHODS

Bacterial and mammalian cell growth conditions. The bacterial strains used in this study are listed in Table 1. The transcriptional fusion reporter strains (27, 29, 37) and the anti-maltose binding protein (MBP)-CpxR antibody (38) were generous gifts from Thomas Silhavy (Princeton University, Princeton, NJ, USA). Bacteria were grown at 37°C in TB7, a medium containing 1% (wt/vol) tryptone and buffered to pH 7.0 with potassium phosphate (100 mM). HepG2 hepatocellular carcinoma cells were a generous gift from Andy Yu (Indiana University, Indianapolis, IN, USA) and were grown in RPMI 1600 medium (Gibco) containing 10% fetal calf serum (Sigma) and 1 mM sodium pyruvate (Sigma) at 37°C with 5% CO_2.

TABLE 1 *E. coli* strains used in this study

Strain	Description	Reference or source
PAD282	MC4100 [*F− araD139* Δ(*argF-lac*)*U169 rpsL150* (Str^r) *relA1 flhD5301 deoC1 ptsF25 rbsR*] λRS88 (*cpxP′-lacZ*)[a]	27
PAD292	PAD282 *cpxR1*::*spc* (spectinomycin insertion in *cpxR* with polar effect on *cpxA*)	27
PAD348	PAD282 *cpxA*::*cam*	DiGiuseppe and Silhavy (Princeton University)
PAD455	PAD282 *cpxA24 zii*::*Tn10*	37
PAD485	PAD282 *nlpE*::*spc*	27
PAD488	PAD282 *cpxP*::*kan*	27
AJW3142	PAD282 *yfiQ*::*kan*	29
TR48	PAD282 *cpxA101 lamBA23D zjb*::Tn*10*Kn	20

[a] Str^r, streptomycin resistant.

β-galactosidase assay. The *E. coli PcpxP′-lacZ* reporter strains were cultured overnight at 37°C in TB7 supplemented with 0.4% glucose. The following day, the cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in 1.7× TB7 with glucose, and 30 μl of the diluted culture were distributed to wells of a 384-well plate containing 20 μl of vehicle or compound and grown at 37°C without shaking. After 5 h, 50 μl of All-in-One β-galactosidase reagent (Pierce), which was diluted 2.5-fold with TB7 medium, was added. The OD_{420}, OD_{550}, and OD_{600} of the wells were measured using a Molecular Devices SpectraMax 384 spectrophotometer, and the Miller units (MU) were calculated as described previously (39). When appropriate, 10% (vol/vol) human AB serum (HyClone) was supplemented. The effect of 10% serum on the activity of the compound was analyzed using a mixed-effects analysis of variance (ANOVA) model.

Z′-factor calculation. Each 384-well plate was loaded with the wild type, Δ*cpxA*, or *cpxA** (PAD455) strain in 22 columns, and medium controls were placed in the remaining 2 columns. The growth conditions were as described for the β-galactosidase assays. The Z′ factor is defined as 1 − [(3 SD of sample + 3 SD of control)/(mean of sample − mean of control)] (SD, standard deviation) and was calculated as described previously (40) from the results from two independent experiments.

Compound collection. We screened 36,000 compounds of a library containing approximately 225,000 compounds obtained from the ChemBridge and ChemDiv collections, housed at the Indiana University Chemical Genomics Core Facility. The library contains nonredundant drug-like small-molecule compounds and provides significant diversity with a multidimensional chemical space. The compounds in the library obey Lipinski's rules for good solubility, absorption, distribution, metabolism, and excretion profiles (41). Commercially available hit compounds, including compound 1, and analogs, including compounds 1a and 1b, were purchased from ChemBridge Hit2Lead, TimTec, or ChemDiv and dissolved in dimethyl sulfoxide (DMSO).

High-throughput screen. A Tecan Freedom EVO robotic liquid handling device was used to transfer 20 μl of 15 μg/ml compound solutions in single wells of columns 1 to 22 of a 384-well plate. The wells in columns 23 and 24 received sterile vehicle. Wild-type bacteria were delivered to all other wells, except for the controls, which contained medium only or the untreated Δ*cpxA* mutant. The growth conditions were as described for the β-galactosidase assays. The final concentration of each compound was 6 μg/ml (approximately 15 μM), and the final concentration of DMSO was 0.5%. Compounds were defined as hits if their treatment met two criteria: (i) it must increase the β-galactosidase activity (in Miller units) of the wild type more than the mean plus three standard deviations of the activity of the untreated wild-type wells, and (ii) it must not decrease growth (in optical density at 600 nm) more than the mean minus three standard deviations of the untreated Δ*cpxA* mutant on the plate. During rescreening of hit compounds and the testing of new compounds, the Miller units were determined at the beginning of the assay and before and after treatment with the β-galactosidase reagent to exclude compounds that are intrinsically yellow in color.

Bacterial viability assays. To determine the compound effect on bacterial viability, cultures were treated with vehicle or compound, and the CFU per milliliter were determined at 0 and 5 h. For the CFU counts, 10 μl of multiple serial dilutions were plated on LB agar and grown at 37°C overnight. Ciprofloxacin (2.5 μM) was used as a control for cell death.

CpxRA pathway mutant assay. The β-galactosidase activity (in Miller units) of the wild-type and *cpxRA* pathway mutant reporter strains was measured in the presence or absence of 0.4% glucose and in the presence of DMSO or 80 μM compound dissolved in DMSO. The fold change was determined by dividing the Miller units obtained in the presence of compound by the Miller units in DMSO only. The fold change data were analyzed using a mixed-model ANOVA with experiment as a random effect to account for within-experiment correlations; they were adjusted for multiple comparisons using Dunnett's procedure.

Detection of phosphorylated CpxR. Wild-type and Δ*cpxA* bacteria were grown overnight in side-arm flasks in TB7 broth without glucose and diluted to an OD_{600} of about 0.1 in 1.7× TB7 supplemented with glucose. To each well of a 384-well plate, 30 μl of this dilution was added to 20 μl of compound 1 (80 μM) dissolved in DMSO or of DMSO alone (192 wells for each strain/treatment). After 5 h of incubation at 37°C, the wells were pooled and harvested by centrifugation. All processing was carried out at 4°C. The cells were washed once in phosphate-buffered saline (pH 7.4) and suspended in 2× Laemmli lysis buffer, according to the bacterial pellet weight. As controls, 10 μM His6-CpxR, expressed and purified as previously described (30), was incubated with 0 or 20 mM AcP at 30°C for 15 min (30). Twenty-microliter and 5-μl aliquots of each cell lysate and AcP treatment, respectively, were separated on a Phos-tag gel, which was prepared according to the procedure described by Lima and colleagues (30) and the manufacturer's protocol, with some modifications. Phos-tag acrylamide was purchased from Wako. The stacking gel contained 4% acrylamide/bis-acrylamide prepared in 350 mM bis-Tris (pH 6.8). The separating gel contained 10% acrylamide/bis-acrylamide, 25 μM Phos-tag acrylamide, and 50 μM $Zn(NO_3)$ prepared in 350 mM bis-Tris (pH 6.8), and was degassed with stirring for 2 min prior to pouring. The gel was run at 4°C in morpholinepropanesulfonic acid (MOPS) buffer (0.1 M MOPS, 0.1 M Tris, 5 mM sodium bisulfite, and 0.1% SDS) for 2 to 3 h at 40 milliamps, and the buffer was refreshed each hour. The gel was washed for 15 min in Towbin transfer buffer containing 1 mM EDTA and then for 30 min in standard Towbin transfer buffer. The proteins were transferred to a polyvinylidene difluoride membrane using a wet transfer method. Tris-buffered saline containing 0.1% Tween 20 (TBST) was used for washing, and TBST supplemented with 5% skim milk was used for blocking and antibody incubations. The membrane was blocked for 1 h and probed overnight at 4°C with 1:10,000 anti-MBP-CpxR antibody. The secondary antibody goat anti-rabbit IgG-horseradish peroxidase conjugate was used at a 1:5,000 dilution for 1 h at room temperature. Densitometry values were determined using Photoshop, and the ratio of CpxR-P to CpxR was analyzed by a one-tailed paired Student's *t* test.

Cytotoxicity assay. To measure cytotoxicity, 20,000 HepG2 (50 μl for the 5-h assay and 100 μl for the 24-h assay) cells were plated per well of tissue culture-treated 96-well plates and allowed to adhere for 3 h. The cells were treated with an equal volume of medium containing either 1% DMSO (vehicle control) or 2× compound, such that the final concentration of DMSO was 0.5%. After 5 and 24 h, lactate dehydrogenase (LDH) release was measured using the CytoTox 96 nonradioactive cytotoxicity assay (Promega), according to the manufacturer's instructions. We determined the percent cell death by adjusting the vehicle-treated and Triton X-100-treated cells to 0 and 100% death, respectively. The 50% inhibitory concentration (IC_{50}) after 24 h of incubation was determined using the GraphPad Prism 6.0 software. The relative cytotoxicities of compounds 1 and 1a were analyzed using a mixed-effects ANOVA model.

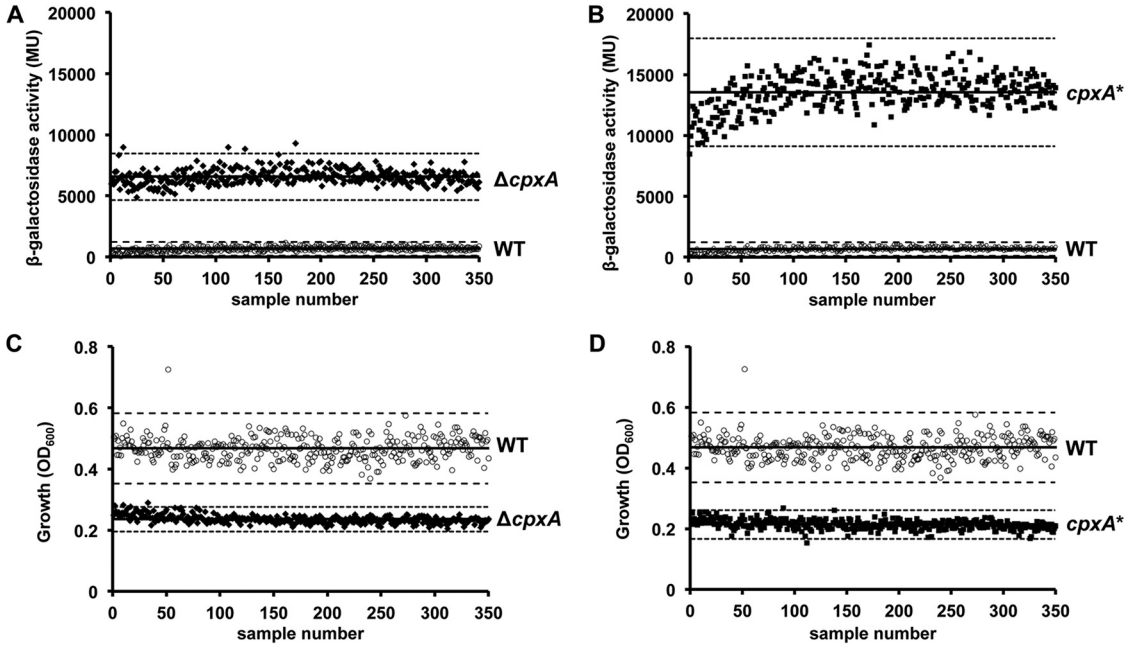


FIG 2 Development of screening assay for CpxA activators. (A and B) β-Galactosidase activity of 352 wells containing the wild type was plotted with 384 wells each of the Δ*cpxA* (A) and *cpxA** (B) mutants. The graphs are representative of the results from 2 independent experiments; average Z′ factors were 0.5 and 0.55 for Δ*cpxA* and *cpxA**, respectively. (C and D) Corresponding growth (optical density at 600 nm) of the wild type was plotted with the Δ*cpxA* (C) and *cpxA** (D) mutants. The graphs are representative of the results from 2 independent experiments. ○, untreated wild type; ◆, Δ*cpxA* mutant; ■, *cpxA** mutant; solid horizontal lines, mean values of the Miller units (A and B) and OD_{600} (C and D) for each strain; dashed lines, 3 standard deviations from the mean.

RESULTS

Development and validation of a HTS assay for CpxR activators. In *E. coli*, *cpxP* is the promoter most highly upregulated by phosphorylated CpxR (25). To detect CpxR activation, we used an isogenic set of *E. coli* reporter strains that contain a chromosomal *cpxP* promoter-*lacZ* fusion (27) (Fig. 1). We developed a 384-well plate assay to detect compounds that activate the reporter in the wild type, using either the Δ*cpxA* or the *cpxA** mutants as standards for *cpxP* transcription and growth. The assay was performed in TB7 broth containing 0.4% glucose, which fosters the formation of AcP. We reasoned that this growth condition would allow the detection of compounds that activate CpxR by augmenting CpxA kinase activity or inhibiting CpxA phosphatase activity.

After 5 h of growth in the glucose-enriched medium, the β-galactosidase activity of the Δ*cpxA* and *cpxA** mutants was approximately 10- and 20-fold greater, respectively, than that of their wild-type parent (Fig. 2A and B). The quality of our assay was assessed by calculating the Z′ factor using the Miller units of the wild-type and the Δ*cpxA* and *cpxA** mutants (Fig. 2A and B). A Z′ factor of ≥0.5 is suitable for HTS (40); our calculated Z′ factor was 0.5 for the Δ*cpxA* mutant and 0.55 for the *cpxA** mutant, indicating that the assay distinguished between baseline and enhanced CpxRA activity. As expected, the Δ*cpxR* mutant exhibited less β-galactosidase activity than that of its wild-type parent (data not shown). On the basis of these data, the first criterion for a compound to be considered a hit was that it must increase β-galactosidase activity (in Miller units) of the wild type more than the mean plus three times the standard deviation of the activity of the untreated wild type.

In interpreting the results of the HTS, we considered the fact that antibiotics indirectly activate CpxRA (42, 43). For example, gentamicin induces protein mistranslation and fosters the translocation of misfolded proteins to the periplasm, activating the system (44). To exclude indirect activators, such as antibiotics, we established growth parameters for the screen. After 5 h, the average optical density at 600 nm of the wild type and the Δ*cpxA* and *cpxA** mutants was 0.5 ± 0.03 (mean ± standard deviation), 0.24 ± 0.01, and 0.21 ± 0.01, respectively (Fig. 2C and D). The growth impairment in the activating mutants suggested that compounds that activate CpxR in the wild type would likely reduce growth to the extent seen in the Δ*cpxA* or *cpxA** mutants. Thus, a second criterion for a compound to be considered a hit is that it may not inhibit the growth of the wild type more than the mean minus three times the standard deviation of the growth of the *cpxA** mutant.

We performed a mock screen in which the wild type was inoculated in 372 wells of the plate, and the Δ*cpxA* and *cpxA** mutants were inoculated in 5 wells each. After 5 h of growth, the reporter activity of the mutants was clearly distinguishable from that of the wild type (Fig. 3). Thus, compounds that activate CpxRA in the wild type should be distinguishable from background.

Whole-cell screen for CpxRA activators. A 36,000-compound small-molecule library was screened at approximately 15 μM for the induction of β-galactosidase activity in the wild-type reporter strain; each compound was tested in a single well. This screen resulted in 340 putative hits. Of 324 compounds available for rescreening, 10 activated the reporter and did not inhibit growth by optical density at 600 nm criterion (Table 2) and satisfied both hit criteria. As controls, we included the bactericidal antibiotic ciprofloxacin and the bacteriostatic antibiotic spectinomycin; both antibiotics activated the reporter in the wild type and caused complete growth inhibition (Table 2). The 10 hit compounds be-

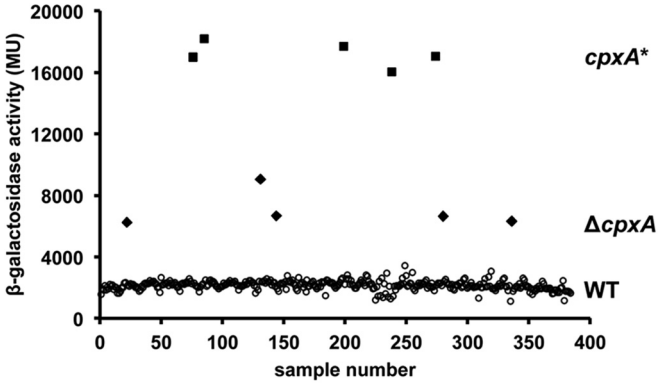


FIG 3 Validation of HTS assay. β-Galactosidase activity of 372 wells of the wild type (○) and 5 wells each of the *cpxA** (◆) and *cpxA* (■) mutants.

longed to three structural groups: six nitroaniline/nitroindole derivatives, three quinolone derivatives, and one furoxan-pyridazine compound that has vasorelaxant activity (45).

We tested the 10 initial hits and 88 commercially available analogs, including 80 nitroanilines/nitroindoles, 17 quinolones, and the furoxan-pyridazine compound. We performed dose-response studies from 1 nM to 100 μM. Most compounds reproducibly activated the reporter in the wild type in concentrations ranging from 10 to 100 μM (data not shown). Importantly, no compounds increased reporter activity in the Δ*cpxR* mutant (data not shown and Fig. 4A), validating the specificity of the screening assay.

The three most potent compounds in terms of *cpxP* transcription that also passed the growth inhibition criterion included two nitroindoles and one quinolone. At concentrations ranging from 5 to 80 μM compound, we measured the viable CFU of the treated wild type and that of the untreated wild type and the Δ*cpxA* and Δ*cpxR* mutants. By quantitative culture, after 5 h, the untreated wild type increased CFU counts by 42-fold, the Δ*cpxR* mutant by 20-fold, and the Δ*cpxA* mutant by 13-fold (data not shown). In contrast, the CFU of the ciprofloxacin-treated wild type decreased almost 10^5-fold (data not shown). At their optimal activating concentrations, the candidate quinolone and one candidate nitroindole caused a 10^2-fold reduction in viable CFU (data not shown), suggesting that they activated the system by killing the bacteria and were not valid candidates. However, in the presence of 40 μM the other candidate nitroindole (6-nitro-2,3,4,9-tetrahydro-1*H*-carbazol-1-amine; catalog no. 5302860; ChemBridge), here referred to as "compound 1," the wild-type CFU increased 4.5-fold (data not shown), suggesting that compound 1 was not activating the system through an antibiotic effect.

Characterization of compound 1. Since compound 1 activated *cpxP* transcription and permitted bacterial growth, we performed dose-response assays in the wild type and Δ*cpxR* mutant; the untreated Δ*cpxA* mutant was included for comparison. A dose-dependent increase in reporter activity was observed in wild-type bacteria but not in the Δ*cpxR* mutant (Fig. 4A). In the presence of the highest concentrations of the compound, reporter activity in the wild type was induced to levels higher than those observed in the untreated Δ*cpxA* mutant (Fig. 4A, dashed lines). In contrast, the antibiotic ciprofloxacin induced reporter activity similar to the lower level of activity observed in the Δ*cpxA* mutant. No additional reporter activity was observed at concentrations of >5 μM ciprofloxacin, likely due to its bactericidal activity (Fig. 4B).

Wild-type bacteria treated with compound 1 mimicked the β-galactosidase activity and growth observed with the Δ*cpxA* mutant; thus, we hypothesized that the compound inhibited CpxA phosphatase activity. If this were true, compound 1 should activate the reporter only in the presence of glucose, CpxA, CpxR, and YfiQ (29). Therefore, we treated the wild-type strain and its isogenic Δ*cpxA*, Δ*cpxR*, and Δ*yfiQ* mutants grown in the presence or absence of glucose with 80 μM compound 1. We calculated the fold change in reporter activity in compound-treated versus untreated wells. Compound 1 maximally activated the reporter in the presence of glucose in the wild type ($P = 0.001$) (Fig. 5A); it was not active in the Δ*cpxA* ($P < 0.0001$), Δ*cpxR*, or Δ*yfiQ* mutants, suggesting that compound 1 likely targeted CpxA. To investigate potential upstream targets, 80 μM compound 1 was tested with the Δ*nlpE* and Δ*cpxP* reporter strains (27). The fold change increase in β-galactosidase activity in the Δ*nlpE* and Δ*cpxP* mutants was not significantly different than the activity in their wild-type parent (data not shown). Taken together, the data suggest that compound 1 either generates a CpxA-activating signal or inhibits CpxA phosphatase activity.

To test the hypothesis that compound 1 inhibits CpxA phosphatase activity, we used the *cpxA** allele *cpxA101* (20). *In vitro*

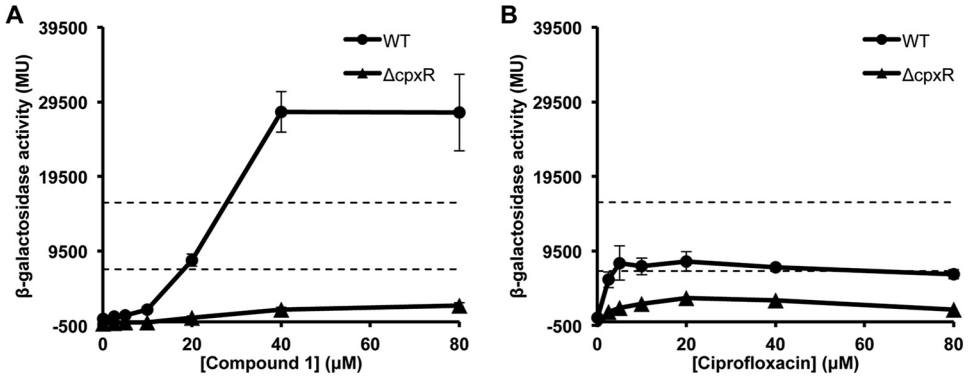
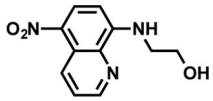
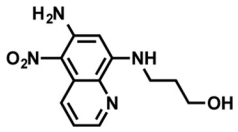
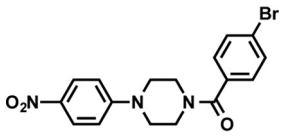
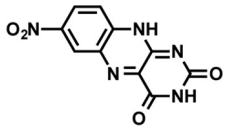
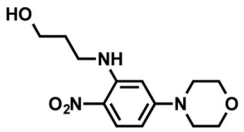
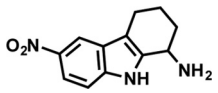
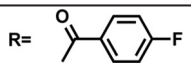
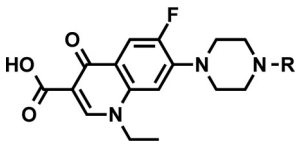
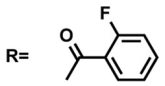
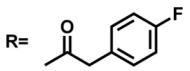
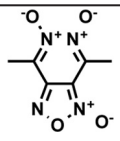


FIG 4 Compound 1 activates CpxRA. Shown are the dose responses of compound 1 (A) and ciprofloxacin (B) with wild-type (WT) and Δ*cpxR* reporter strains. The reporter activity was determined after 5 h of incubation with increasing concentrations of compound 1 or ciprofloxacin. Data are means ± standard deviations from three independent experiments. The dashed lines represent the mean plus and minus three standard deviations of the untreated Δ*cpxA* mutant.

TABLE 2 Structure, compound-induced activity (Miller units), and growth (OD_{600}) of compound-treated cells relative to wild type in the confirmatory assay

Hit no.	Structural group[a]	Structure	Supplier ID[b]	Fold change relative to WT in[c]: MU	Fold change relative to WT in[c]: OD_{600}
1	A		CB5139225	2.12	1.03
2			CB5139230	4.00	1.00
3			CB5221072	2.13	1.13
4			CB5315987	2.26	1.10
5			CB5406089	2.31	1.03
6			CB5302860	27.64	0.69
7	B		CD5076-4234	5.57	0.69
8		 	CD5076-2815	4.16	1.15
9			CD5081-0145	12.29	0.82
10	C		CB5314731	4.89	0.77
		Ciprofloxacin		9.05	0.20
		Spectinomycin		10.61	0.21

[a] A, B, and C are the 3 classes of hits identified in the high-throughput screen: nitroaromatics, quinolones, and the furoxan-pyridazine compound, respectively.
[b] ID, identification; CB, ChemBridge; CD, ChemDiv.
[c] MU, Miller units; WT, wild type.

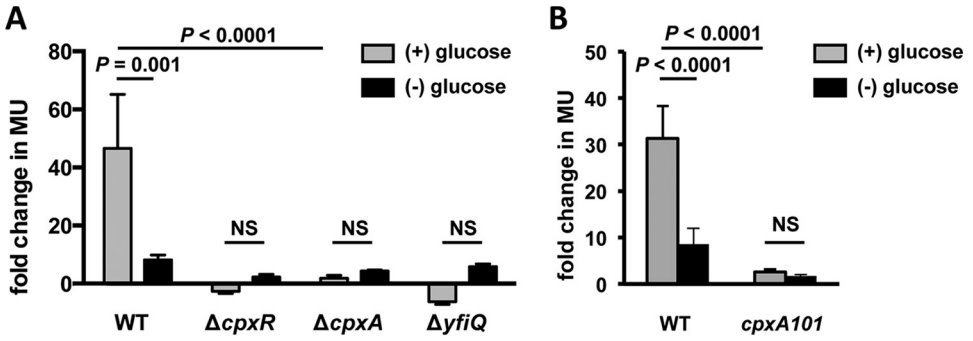


FIG 5 Compound 1 activity requires glucose, CpxA, CpxR, YfiQ, and CpxA phosphatase activity. The fold changes in reporter activity after treatment with 80 μM compound 1 in the presence or absence of glucose in wild type (WT) and the Δ*cpxA*, Δ*cpxR*, and Δ*yfiQ* mutants (A) or in WT and the *cpxA101* mutant (B) are shown. For each strain, the fold change was calculated by dividing the β-galactosidase activity (in Miller units) of the compound-treated wells by the Miller units of the untreated wells. The data are the average and standard deviation from three independent experiments.

phosphorelay assays showed that CpxA101 retains autokinase and CpxR kinase activity but lacks CpxR-P phosphatase activity (20). We treated the wild-type strain and the *cpxA101* mutant in the presence or absence of glucose with 80 μM compound 1 and calculated the fold change in reporter activity in compound-treated versus untreated wells. As observed previously, the compound maximally activated the reporter in wild-type bacteria in the presence of glucose, whereas significantly less activity was observed in the absence of glucose ($P < 0.0001$) (Fig. 5B). In contrast, the compound-induced reporter activity did not differ in the presence or absence of glucose in the *cpxA101* mutant (Fig. 5B). The compound-induced reporter activity of the wild type was significantly higher than that in the *cpxA101* mutant ($P < 0.0001$) (Fig. 5B). These results also suggested that compound 1 predominantly acts by inhibiting CpxA phosphatase activity.

Compound 1 causes accumulation of phosphorylated CpxR. We reasoned that if compound 1 inhibits CpxA phosphatase activity, it should induce an increase in CpxR-P levels. Lima and colleagues (30) recently used Phos-tag SDS-PAGE to detect CpxR-P in *E. coli* grown in the presence of 0.4% glucose. To test if compound 1 induces CpxR-P accumulation, we cultured the wild type in the presence of 0.4% glucose and with or without 80 μM compound 1. As controls, we included recombinant CpxR that was untreated or treated with 20 mM AcP, along with the untreated Δ*cpxA* mutant grown in the presence of glucose. We harvested the cells after 5 h, prepared cell lysates, separated proteins by Phos-tag SDS-PAGE, and detected endogenous CpxR and CpxR-P by Western immunoblot with anti-MBP-CpxR antiserum. As shown in Fig. 6A, the treatment of CpxR with AcP increased the fraction of CpxR-P. Treatment with compound 1 induced an accumulation of CpxR-P levels in wild-type cells equivalent to that in the activated Δ*cpxA* mutant. Total CpxR levels were higher in the Δ*cpxA* mutant and treated wild type, consistent with the fact that CpxR-P positively autoregulates its transcription. To accurately compare the samples, the two CpxR fractions were quantified by densitometry. Compared to the untreated wild type, compound 1 treatment trended toward increasing the ratio of CpxR-P to CpxR in the wild type by 3-fold ($P = 0.057$) (Fig. 6B).

Activity of compound 1 derivatives. To determine whether the nitro and amine moieties of compound 1 were necessary for activity, we obtained two derivatives: compound 1a (2,3,4,9-tetrahydro-1*H*-carbazol-1-amine; catalog no. 8019-9961; ChemDiv), which lacks the nitro group, and compound 1b (3-nitro-5,6,7,8,9-pentahydro-4aH-carbazole; catalog no. ST024298; TimTec), which lacks the amine group (Fig. 7A). The activity of the three compounds was evaluated in the wild-type reporter strain in the presence of 0.4% glucose. The activity of compound 1a was not significantly different than that of compound 1, whereas compound 1b elicited little reporter activity ($P < 0.005$ versus compound 1 and $P = 0.007$ versus compound 1a) (Fig. 7B). Thus, activation of the reporter depended on the presence of the amine group and not the nitro group. Using the maximum re-

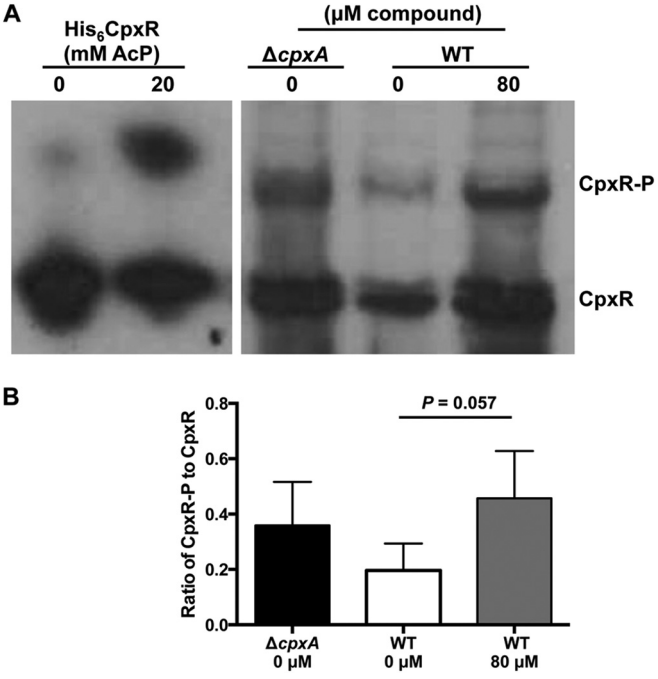


FIG 6 Compound 1 induces phospho-CpxR accumulation. (A) Composite representative Western blot of His_6CpxR incubated with 0 or 20 mM AcP, and the Δ*cpxA* mutant and WT grown in medium with 0.4% glucose and 0 or 80 μM compound. Note that His_6CpxR migrates slower than native CpxR. (B) The ratio of CpxR-P to CpxR was determined using densitometry; the data are the mean and standard deviation results from 4 independent experiments.

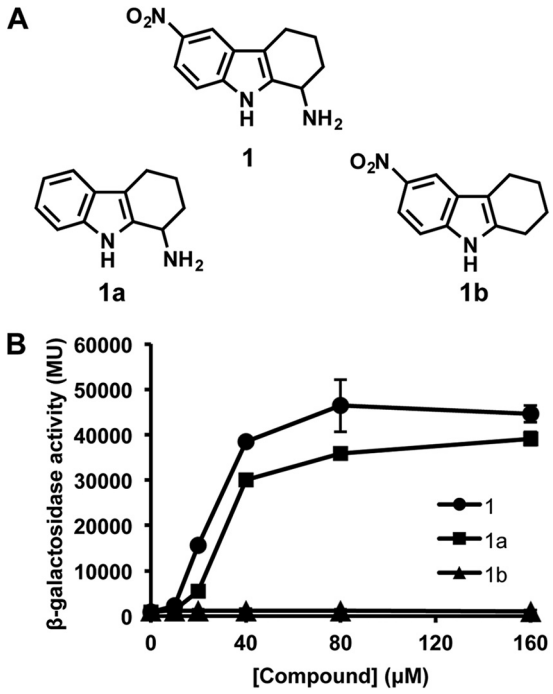


FIG 7 Activity of compound 1 and derivatives. (A) Structure of compound 1 and derivatives lacking the nitro group (1a) or the amine group (1b). (B) Reporter activity in wild-type *E. coli* after 5 h of incubation with increasing concentrations of compounds 1, 1a, and 1b. The data are the mean and standard deviation of the results from three independent experiments. The activity of compounds 1 and 1a was not significantly different, whereas the activity of compound 1b was reduced compared to that of compound 1 ($P < 0.005$).

porter activity induced by each compound, we estimate the 50% effective concentrations (EC_{50}s) to be 25 and 30 μM for compounds 1 and 1a, respectively. By quantitative culture, wild-type bacteria grew 5.3-fold in the presence of 40 μM compound 1a. Thus, the CpxRA activation induced by compound 1a mimics that of compound 1.

Activity in surrogate mammalian systems. To determine the potential utility of this class of compounds in a mammalian model, we next assessed their activity in the presence and absence of 10% human AB serum. Whereas the presence of serum trended toward reducing the activity of compound 1 ($P = 0.058$) (Fig. 8A), the activity of compound 1a was not significantly reduced in the presence of serum (Fig. 8B). These results suggested that compound 1a could be active *in vivo*.

After validating the compound activity in serum, we next assessed their effects on mammalian cell viability. The cytotoxicity of compounds 1 and 1a was determined using HepG2 hepatocellular carcinoma cells. HepG2 cells were treated with increasing concentrations of compounds 1 and 1a for 5 and 24 h. Cell viability was determined by measuring LDH release via 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction. After 5 h, compounds 1 and 1a caused <10% and 5% cell death, respectively ($P = 0.21$). After 24 h, compound 1 had an IC_{50} of 63 μM, whereas compound 1a was less cytotoxic, with an IC_{50} of 139 μM ($P = 0.03$) (Fig. 8C). Thus, the cytotoxicity of compound 1 after 24 h of incubation was reduced with the removal of the nitro group, consistent with the fact that nitro groups are often implicated in cytotoxicity (46).

DISCUSSION

Because bacterial 2CSTS frequently have essential functions and lack mammalian homologs, there has been considerable interest in targeting 2CSTS for antimicrobials (13–15, 18, 47). The mode of action of reported 2CSTS-targeted compounds is the inhibition of SK activity or interference with the binding of the RR to its promoter (16, 18, 48). To the best of our knowledge, this is the first report describing a compound that activates, rather than inhibits, a 2CSTS.

In this study, we developed and validated a novel HTS based on genetic activation of the *cpxRA* system. We established growth and reporter activity cutoffs using the untreated wild type and Δ*cpxA* and *cpxA** mutants grown in the presence of glucose. As shown in Fig. 2 and 3, reporter activity in the Δ*cpxA* and *cpxA** mutants was readily distinguishable from the wild-type reporter activity, providing a robust HTS assay. The facile nature of the β-galactosidase assay makes this a broadly applicable screening strategy.

Most biochemical screens performed in our core facility have typical hit rates of 0.1 to 0.2%. Our limited screen of 36,000 compounds yielded 340 hits, 10 of which were confirmed, giving a hit rate of 0.027%. Performing the screen in wild-type *E. coli* may account for the low hit rate, due to limited uptake and/or efflux of the small molecules. The archetype of multidrug efflux pumps in *Enterobacteriaceae* is the AcrAB-TolC system; inactivation of these transporters increases susceptibility to multiple antibacterial agents (reviewed in references 49 and 50). By allowing more compounds to reach their intracellular target, *E. coli* mutants lacking AcrAB or TolC are frequently used in HTS assays to increase the hit rate. However, the deletion of *tolC* activates the CpxRA system in *E. coli* (51). Similarly, CpxRA is activated by the deletion of the efflux pump genes *mtrC* in *H. ducreyi* (52) and *vexAB* or *vexGH* in *Vibrio cholerae* (53). Thus, wild-type bacteria must be used to identify CpxRA activators.

A major advantage of our cell- and reporter-based screening assay is its ability to identify compounds that specifically activate the CpxRA system. In theory, hit compounds might target different members of the Cpx pathway (i.e., CpxP, NlpE, CpxA, and CpxR), the acetyltransferase YfiQ, or enzymes involved in central metabolism. None of the hit compounds increased reporter activity in the Δ*cpxR* mutant (data not shown and Fig. 4A), confirming the specificity of our assay. The availability of an isogenic set of *cpxRA* pathway mutants bearing the reporter allowed us to infer the likely mechanism of action of the hits. Using these mutants, we demonstrated that compound 1 requires *cpxR*, *cpxA*, *yfiQ*, and glucose for activity but does not require *nlpE* or *cpxP* (Fig. 5A). Furthermore, the compound was not active in the *cpxA101* mutant, which lacks phosphatase activity (Fig. 5B). Taken together, the data suggest that compound 1 targets CpxA and likely inhibits its phosphatase activity.

Δ*cpxA*-activating mutants in *E. coli* and *Yersinia pseudotuberculosis* accumulate CpxR-P (30, 54). We detected a trend for a compound-induced increase in the phosphorylated fraction of CpxR to levels comparable to those in the untreated Δ*cpxA* mutant (Fig. 6). Increased CpxR-P levels are consistent with the observed increased transcription from the *cpxP* promoter. Although the genetic and cell-based assays described here provide preliminary identification of targets, biochemical characterization is necessary to elucidate the mode of action of the compound. *In vitro*

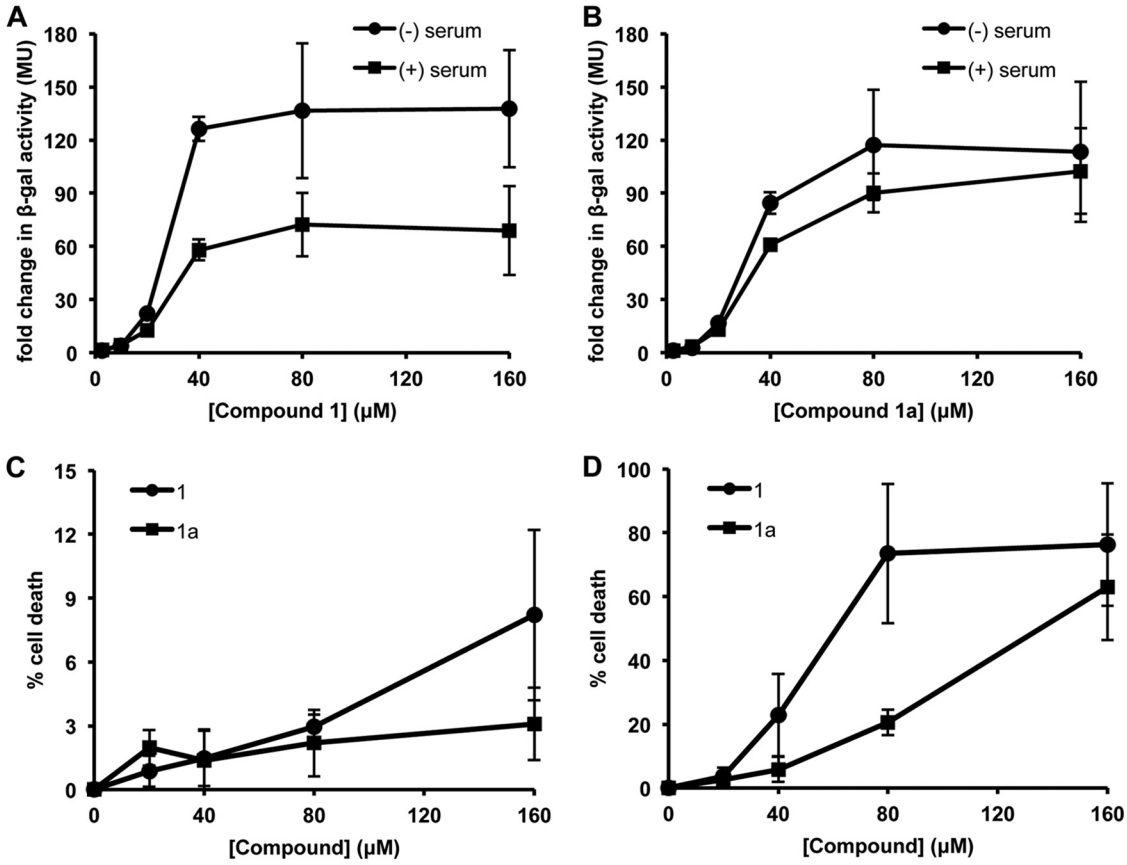


FIG 8 Compound activity in 10% serum and cytotoxicity with HepG2 cells. (A and B) Fold change in reporter activity of wild type after treatment with increasing concentrations of compound 1 (A) or 1a (B) in the presence or absence of 10% human AB serum. For both conditions, the fold change was calculated by dividing the β-galactosidase (β-gal) activity (in Miller units) in the compound-treated wells by the Miller units of the untreated wells. The data are the mean and standard deviation values from three independent experiments. The presence of serum trended toward reducing compound 1 activity ($P = 0.058$), whereas the activity of compound 1a was not significantly affected by the presence of serum. (C and D) Cytotoxicity of compounds 1 and 1a with HepG2 cells. Hepatocellular carcinoma HepG2 cells were treated with compounds 1 and 1a for 5 h (C) and 24 h (D). Cell viability was determined by LDH release. The data are the mean and standard deviation values from four independent experiments. The cytotoxicity caused by compound 1a was significantly less than that by compound 1 after 24 h of incubation ($P = 0.03$). Note the scale-adjusted ordinate axis for panels C and D.

phosphorelay assays are under way to elucidate the effect of compound treatment on CpxA enzymatic activity.

The activity of a compound in the presence of serum is important when considering potential efficacy *in vivo*. The presence of 10% human serum reduced compound 1 activity by approximately 50%. In contrast, compound 1a retained full activity in the presence of serum (Fig. 8A and B). Thus, compound 1a does not appear to nonspecifically bind to or react with proteins found in serum. Since the compounds retained activity in serum, it was appropriate to examine cytotoxicity. HepG2 hepatocellular carcinoma cells were chosen as the model because the liver metabolizes most drugs. As nitro groups are known to be cytotoxic (46), it is not surprising that removal of the nitro group relieved the cytotoxicity of compound 1 >2-fold (Fig. 8C and D). Thus, compound 1a is an optimized first-generation derivative: it activates CpxRA, retains complete activity in serum, and is less cytotoxic than compound 1. In a 5-h assay, 80 μM compound 1a maximally activated CpxRA and caused negligible cell death (2.2%). Thus, medicinal optimization is under way to develop compound 1a into a highly potent lead compound.

A consideration for the clinical utility of *cpxRA* activators is the recent implication of the requirement of *cpxRA* activation in the mode of action of bactericidal antibiotics. Some studies suggest that bactericidal antibiotics kill *E. coli* through a "final common death pathway," which is mediated in part by *cpxRA* activation and is inhibited by the deletion of *cpxA* or *cpxR* (42, 43, 55). However, Mahoney and Silhavy (44) showed that *cpxR* is not required for killing by bactericidal antibiotics and that an *E. coli cpxA** mutant is as susceptible to ampicillin and norfloxacin as the wild type (44). However, a *cpxA** mutant is less susceptible to gentamicin than the wild type, perhaps because the preactivation of CpxR by CpxA* prevents membrane damage induced by the misfolded proteins (44). Although an *E. coli* Δ*cpxA* mutant is less susceptible than the wild type to 5 μg/ml gentamicin, the *cpxA** mutant is as susceptible as the wild type to 15 μg/ml gentamicin (43), a level that is exceeded clinically with once-daily dosing regimens. Thus, depending on the level of activation and the antibiotic concentration, activating compounds may interfere with aminoglycosides but not with other antibiotics.

In conclusion, we developed a robust screening strategy to identify and characterize CpxRA activators. We plan to extend our HTS and confirmatory screening assay to identify more potent

activators of the *cpxRA* system. As CpxA is highly conserved in *Enterobacteriaceae*, a compound that targets CpxA would likely have a broad spectrum of activity. Since *cpxA** mutants show the highest level of CpxR activation, compounds that enhance CpxA kinase activity would likely be more potent than a CpxA phosphatase inhibitor. Compound 1a and other activators will serve as valuable probes to further study the role of *cpxRA* in pathogenesis and to address the utility of *cpxRA* activation as a nontraditional antimicrobial strategy. We have named this new class of drugs astabiotics (antimicrobial signal transduction activator-biotics). If our approach is successful, astabiotics could be sought for different targets in other bacterial pathogens and potentially revolutionize the field. For example, *Pseudomonas aeruginosa* contains a 2CSTS, AmgRS, which, although not homologous to CpxRA, controls a similar set of genes to combat envelope stress (56). Inhibitors of AmgRS are being studied to enhance the efficacy of aminoglycosides; however, activators of AmgRS might have antimicrobial effects *in vivo*.

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