Antibiotic resistance and the threat of a postantibiotic era continue to be a growing global problem. Furthermore, the discovery and/or availability of novel classes of antimicrobial agents is in retreat (1, 2). Contributing to the latter, traditional drug discovery is a highly inefficient, costly, and challenging process. As a result, systematic screening of nonantibiotic Food and Drug Administration (FDA)-approved drugs for other indications in humans offers a rapid alternative for novel antimicrobial drug discovery (3, 4). Such drugs potentially have antibiotic-like activity, modulate bacterial virulence, or regulate host genes necessary for bacterial replication and hence may aid in pathogen clearance (3, 4). In recent years, increased focus has been placed on characterizing host mechanisms/pathways exploited by bacteria during pathogenesis. Drugs able to block these pathways represent novel therapeutic options and also reduce the likelihood of the development of resistance, unlike antibiotics (5–7). Some recent studies utilized such drug-repurposing approaches to identify both novel bactericidal and host-directed drugs as potential therapeutics against pathogens, such as Ebola virus, Borrelia burgdorferi, Coxiella burnetii, and Legionella pneumophila (5, 8–10).

We initiated this study by focusing on the highly virulent pathogen *Yersinia pestis*, the causative agent of plague. Plague manifests itself in three forms, namely, bubonic, septicemic, and pneumonic (11), with the last two having a very high mortality rate (approaching 100%) and a narrow window for medical intervention (12, 13). The organism is transmitted to humans through either the bite of an infected flea or inhalation of the organism and has been responsible for three major human pandemics throughout history and for over 200 million deaths worldwide (11, 13, 14). Today, this endemic bacterium remains a prevalent public health threat in many regions of the world, with a recent outbreak reported in Madagascar (2014) as well as fatal cases of septicemic and pneumonic plague reported in Colorado (2015) (15, 16). As such, *Y. pestis* has been classified as a reemerging pathogen by the World Health Organization (WHO) and as a tier 1 select agent by the Centers for Disease Control and Prevention (CDC), because of its potential to be weaponized in biological warfare (12, 17). Plague is treatable with antibiotics, and levofloxacin (Levaquin) and moxifloxacin (Avelox) were approved by the FDA in 2012 and 2015, respectively (13, 18). However, such antimicrobials must be administered within 20 to 24 h after the onset of symptoms to be effective, meaning that, in many cases, patients have to be treated before there is a definitive diagnosis (12, 19). The value of antibiotic treatment is further diminished because multiple-antibiotic-resistant *Y. pestis* strains have been isolated from plague patients in Madagascar and/or genetically engineered for possible use as a bioweapon (20–22).
In order to identify potential novel therapeutics, we conducted a screen of 780 FDA-approved drugs to assess macrophage viability following *Y. pestis* CO92 infection. Although not an exact clinical model, macrophages are an essential component of innate immunity and the first line of defense for numerous infections, including those with the facultative intracellular pathogen *Y. pestis* (11, 23, 24), making this approach a rapid and effective way to identify lead therapeutic compounds for further studies in *in vivo* models. By *in vitro* screening, we reproducibly, through two independent experiments performed in duplicate, identified 94 drugs significantly effective at preventing macrophage cytotoxicity during *Y. pestis* infection. From the 780 screened drugs, a total of 17 were prioritized, based on *in vitro* screening methods, and assessed in a murine model of pneumonia plaque. The following three drugs increased animal survival: trifluoperazine (TFP), an antipsychotic of the phenothiazine class; doxapram (DXP), a breathing stimulant; and amoxapine (AXPN), a tricyclic antidepressant. Interestingly, these three drugs were shown to have no impact on bacterial growth or expression/production of type 3 secretion system (T3SS) effectors and exhibited high MIC values which would be difficult to achieve in human plasma.

To demonstrate the potential for broad applicability of the novel drugs, the therapeutic potential of TFP, which was the most efficacious drug in terms of animal survival in the *Y. pestis* infection model, was tested in murine models of *Salmonella enterica* serovar Typhimurium and *Clostridium difficile* infections. Multi-drug-resistant *Salmonella* strains represent an inevitable consequence of the use of antibiotics in food-producing animals or for human treatment (1, 25–27). *C. difficile* is an emerging worldwide public health problem and the leading cause of nosocomial antibiotic-associated diarrhea in the United States (28). Patients experience repeated episodes of recurrent diarrhea and require antibiotic treatment for prolonged periods (29). The emergence of hypervirulent and antibiotic-resistant strains has increased the risk of developing pseudomembranous colitis worldwide (29). As such, both *S. Typhimurium* and *C. difficile* represent emerging public health problems on account of their multiple-drug resistance, underscoring the need for the rapid development of alternative therapeutics.

For both the *S. Typhimurium* and *C. difficile* infection models, TFP significantly increased the survival of infected mice. Thus, the drugs identified through the high-throughput screen may have broad applicability, as numerous pathogens may rely on similar mechanisms to modulate bacterial virulence or host pathways. Such new drugs may be highly effective against multiple-antibiotic-resistant pathogens, thus countering the current and growing problem of antimicrobial ineffectiveness.

**MATERIALS AND METHODS**

**Bacterial strains and cell culture.** The highly virulent *Y. pestis* CO92 strain was obtained from the Biodefense and Emerging Infections (BEI) Research Resources Repository, Manassas, VA. The *Y. pestis* CO92-lux strain, which harbors the luciferase gene, was generated in our previous study (30). *Y. pestis* strains were grown in heart infusion broth (HIB) (Difco; Voigt Global Distribution, Inc., Lawrence, KS) at 28 or 37°C with constant shaking at 180 rpm. All experiments with *Y. pestis* strains were performed in the CDC-approved select agent laboratory at the Galveston National Laboratory (GNL), University of Texas Medical Branch (UTMB).

The *S. Typhimurium* 14028 strain was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The organism was grown in Luria-Bertani (LB) broth at 37°C with constant shaking at 180 rpm.

The *C. difficile* VPI 10463 strain was also obtained from ATCC. The organism was grown anaerobically in a cooked meat medium (Fluka, St. Louis, MO) as previously described (31, 32).

RAW 264.7 murine macrophage cell lines (ATCC) were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum supplemented with 1% l-glutamine (Cellgro, Manassas, VA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% CO₂.

**Reagents.** The Screen-Well FDA-approved drug library V2, consisting of 780 compounds, was provided as 10 mM stock solutions in dimethyl sulfoxide (BML-2843-0100; Enzo Life Sciences, Albany, NY). Sterile, injectable formulations of levofloxacin, doxapram (DXP), haloperidol, carboplatin, dihydroergotamine mesylate, and promethazine were purchased from the UTMB Pharmacy. Tablet formulations of zarifulin, mesalamine, colchicine, and aspirin were also purchased from the UTMB Pharmacy. Amoxapine (AXPN), trifluoperazine (TFP), desogestrel, ethinyl estradiol, c-armamic acid, apomorphine, and pantoprazole were purchased from Sigma-Aldrich (St. Louis, MO) in a dry powder form. Epinastine, also in dry powder form, was purchased from Abcam (Cambridge, MA). MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide] and detergent solution were purchased from Promega (Madison, WI).

**Screening for macrophage cell viability.** Murine RAW 264.7 cells were seeded in 96-well microtiter plates at a concentration of 2 × 10⁴ cells/well (in 100 μl/well) to form confluent monolayers.

**Pretreatment screens.** Macrophages were incubated with drugs at 33 μM (each) 2 h prior to infection (see Fig. S1 in the supplemental material). Plates were then infected with *Y. pestis* CO92 at a multiplicity of infection (MOI) of 100, centrifuged at 1,250 rpm for 10 min to promote bacterial contact with the host cells, and incubated at 37°C and 5% CO₂ for 60 min. The wells were then washed with phosphate-buffered saline (PBS), and extracellular bacteria were killed by the addition of 50 μg/ml of gentamicin to each well for 60 min at 37°C and 5% CO₂. The wells were then washed with PBS, and macrophages were maintained in medium containing a maintenance concentration (10 μg/ml) of gentamicin for 12 h before performance of the MTT assay (see below).

**Posttreatment screens.** Following infection and treatment with gentamicin (2 h postinfection [p.i.]), drugs were added at 33 μM (each) to macrophages (see Fig. S1 in the supplemental material), and the mixtures were incubated for 12 h at 37°C and 5% CO₂. Reduction of MTT was used as an index of cell viability following the protocol outlined by ATCC. Briefly, the MTT reagent was added to the microtiter plate wells (10 μl/well) to form confluent monolayers, and the plates were incubated for 12 h at 37°C and 5% CO₂.

**Screening for inhibition of *Y. pestis* CO92 intracellular survival.** The *Y. pestis* CO92-lux strain was grown in HIB overnight to saturation at 28°C. RAW 264.7 macrophages were seeded in 96-well plates at a concentration of 2 × 10⁴ cells/well for confluence. Plates were then infected with *Y. pestis* CO92-lux at an MOI of 250 in DMEM, centrifuged, and incubated at 37°C and 5% CO₂. Reduction of MTT was used as an index of cell viability following the protocol outlined by ATCC. Briefly, the MTT reagent was added to the microtiter plate wells (10 μl/well), and the plates were incubated for 12 h at 37°C and 5% CO₂. At 0, 4, 8, and 12 h p.i., luminescence was measured in a Spectramax M5e microplate reader (Molecular Devices, Sunnyvale, CA).

**Testing lead drugs in *Y. pestis* CO92 pneumonic plague model.** All of the animal studies with *Y. pestis* were performed in an animal biosafety level 3 (ABSL-3) facility under an approved Institutional Animal Care and Use Committee (IACUC) protocol (UTMB). Six- to 8-week-old female Swiss Webster mice (17 to 20 g), purchased from Taconic Laboratories (Germantown, NY), were anesthetized by the intraperitoneal (i.p.) route.
with a mixture of ketamine and xylazine and subsequently challenged intranasally (i.n.) with 10 500% lethal doses (LD50) (1 LD50 = 500 CFU) of wild-type (WT) Y. pestis CO92. Immediately following infection, mice were dosed through either the i.p. or oral route with one of 17 lead drugs (identified through the in vitro screens described above) at concentrations ranging from 0.025 to 150 mg/kg of body weight (see Table S1 in the supplemental material). Dosing occurred once at the time of challenge or once every 24 h for up to 6 days. Beginning at 24 h p.i., an additional group of mice were dosed with 5 mg/kg levofloxacin administered i.p. at 24-h intervals for 3 days to serve as a positive control for therapeutic treatment. Mice were assessed for morbidity and/or mortality as well as clinical symptoms for the duration of each experiment (up to 21 days p.i.).

For a combinational study with TFP and levofloxacin, mice were dosed with TFP through the i.p. route at a concentration of 1.5 mg/kg at the time of infection, while levofloxacin (0.25 mg/kg; subinhibitory dose) was administered i.p. at 24 h p.i. and subsequently at 24-h intervals for 3 days. Saline, TFP (1.5 mg/kg)-only, and levofloxacin (0.25 mg/kg and 5 mg/kg)-only groups of mice were used as controls and dosed as described above.

Screening for dose–response effects on macrophage viability following treatment with TFP, DXP, and AXPN and infection with Y. pestis CO92. Following the posttreatment protocol outlined above, murine RAW 264.7 cells were seeded in 96-well microtiter plates at a concentration of 2 × 10^4 cells/well (in 100 μl/well) to form confluent monolayers. Macrophages were then infected with Y. pestis CO92 at an MOI of 100 and subsequently incubated with gentamicin (at 2 h p.i.). TFP, DXP, and AXPN were then added at 1, 10, 20, 30, and 50 μM to macrophages and incubated for 12 h at 37°C and 5% CO2. Reduction of MTT was used as an index of cell viability, and absorbance values at 570 nm were measured in a Spectramax M5e microplate reader.

**Growth kinetics and sensitivity of Y. pestis CO92 to TFP, DXP, and AXPN.** Overnight cultures of Y. pestis CO92, grown in HIB at 28°C, were normalized to the same absorbance by measuring the optical density at 600 nm (OD600). Subcultures were then inoculated into 20 ml of HIB containing in 125-ml polycarbonate Erlenmeyer flasks with HEPA-filtered tops and containing 33 μM TFP, DXP, AXPN, levofloxacin (positive control), or PBS (negative control). The cultures were incubated at 37°C with agitation, and samples for absorbance measurements were taken at the indicated time points. For MIC determinations, the broth macrodilution method was utilized (33). Briefly, Y. pestis CO92 was grown to saturation at 28°C and adjusted to a 0.5 McFarland standard before addition of the same volume of the culture to serial dilutions of each drug (the highest concentration tested was 100 μg/ml) in 5 ml of HIB. Cultures were grown for 24 h with agitation at 37°C. Bacterial growth with PBS or with levofloxacin served as a negative or positive control, respectively.

**Evaluation of Y. pestis CO92 T3SS function and plasmomogen activator (Pla) protease activity in response to TFP, DXP, and AXPN.** For Western blot analysis of T3SS effectors, overnight cultures of Y. pestis CO92, grown in HIB at 28°C, were diluted 1:20 in 5 ml HIB supplemented with 5 mM EGTA to lower the calcium concentration. TFP, DXP, or AXPN was then added at 33 μM, and the culture was incubated at 28°C for 2 h before being shifted to 37°C (to activate the T3SS) for an additional 3 h of growth. After centrifugation, the cell pellets were dissolved in SDS-PAGE buffer and analyzed by immunoblotting using antibodies to YopE and LcrV (Santa Cruz Biotechnology, Santa Cruz, CA). Aliquots (1 ml) of supernatants were precipitated by 20% (vol/vol) trichloroacetic acid (TCA) on ice for 2 h. The TCA precipitates were then dissolved in SDS-PAGE buffer and analyzed by immunoblotting using antibodies to YopE. The anti-DnaK monoclonal antibody (Enzo) was employed for analysis of cell pellets that ensure similar numbers of bacteria were used across testing conditions.

For measurement of Pla protease activity, WT CO92 was plated on a HIB agar plate from a ~80°C glycerol stock and incubated at 28°C for 36 h. A single colony was replated on a fresh HIB agar plate and incubated at 28°C for 20 to 22 h. Bacteria were then suspended in HIB and incubated with 33 μM TFP, AXPN, or DXP, or with PBS as a control, and grown overnight. Cultures were centrifuged, washed twice, and resuspended in PBS with a 33 μM concentration of the respective drug to obtain a final OD600 of 0.25 in a spectrophotometer (SmartSpec 300; Bio-Rad). For each sample, 50-μl suspensions (3.1 × 10^6 CFU/well) were added to wells of a black microtiter plate (Costar Corning Inc., Corning, NY) in triplicate. Samples containing only PBS and each drug were also analyzed for any drug autofluorescence. The hexapeptide substrate DABCYL-Arg-Arg-Ile-Asn-Asg-Glu (EDANS)-NH2 synthesized on Siebler amide resin (34), was added to the wells at a final concentration of 2.5 μg/50 μl. The kinetics of substrate cleavage by Pla was measured every 10 min for 3 h by a fluorometric assay (excitation/emission wavelengths, 360/460 nm) at 37°C on a BioTek Synergy HT spectrophotometer (BioTek Instruments Inc., Winooski, VT).

**Testing TFP as a therapeutic in models of S. Typhimurium and C. difficile infections.** All of the animal studies with S. Typhimurium and C. difficile were performed in an ABSL-2 facility under approved IACUC protocols (UTMB). For S. Typhimurium, 6–8-week-old female Swiss Webster or BALB/c mice were dosed with 1.5 mg/kg TFP or saline by the i.p. route 3 h prior to infection, as TFP has previously been reported to increase animal survival in a septicemic model of Salmonella infection when dosed prior to infection (35). Following drug treatment, mice were challenged i.p. with WT S. Typhimurium (1.0 × 10^8 CFU [1,000 LD50]) (36). Following infection, an additional group of mice was dosed at 24-h intervals with either 5 mg/kg or 0.25 mg/kg levofloxacin for up to 3 days to serve as a therapeutic control. Mice were assessed for morbidity and/or mortality as well as clinical symptoms over the duration of the experiment (14 days p.i.).

For the C. difficile infection model, female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under specific-pathogen-free conditions. At 8 weeks of age, mice were administered an antibiotic cocktail in the drinking water (colistin, 850 U/ml; gentamicin, 0.035 mg/ml; kanamycin, 0.4 mg/ml; metronidazole, 0.215 mg/ml; and vancomycin, 0.045 mg/ml) for 3 days to sensitize animals to infection and then were switched to regular water (31). Two days later, mice received a single dose of clindamycin (32 mg/kg) via i.p. injection to disrupt the normal intestinal microbiota, allowing C. difficile colonization. The next day, 3 h prior to infection, mice were dosed with 1.5 mg/kg TFP or saline by the i.p. route and were infected, following the drug treatment, with 10^8 spores of C. difficile (strain VPI 10463) by oral gavage.

Briefly, C. difficile was grown under anaerobic conditions in cooked meat medium for ~7 days at 20 to 26°C to allow for sporulation. Spores were then separated from vegetative cells via density gradient centrifugation (33), heat shocked at 56°C for 20 min to kill any remaining vegetative cells, and then centrifuged. Resultant pellets were resuspended at 1 × 10^6 cells/ml in sterile saline and enumerated on C. difficile selective agar plates containing 7% horse blood, α-cyclodextrin, and cefoxitin. Spore suspensions with a purity of >99% were stored at 20°C. Following infection, an additional group of mice was dosed with 20 mg/kg vancomycin at 24-h intervals for 5 days p.i. to serve as a therapeutic control. All mice were monitored daily for signs of infection, including weight loss, presence of diarrhea, hunched posture, and prolonged lethargy. At necropsy, ceca and colons were removed and processed for histological analysis to assess the degree of inflammation and damage to the mucosa.

**Growth kinetics and sensitivity of S. Typhimurium to TFP.** Overnight cultures of S. Typhimurium, grown in LB broth at 37°C, were subcultured to an OD600 of 0.1. Cultures were then inoculated with 33 μM TFP or levofloxacin (positive control) or with PBS (negative control) and incubated at 37°C with shaking. Samples for OD600 measurements were taken at the indicated time points. For MIC determinations, the broth macrodilution method was utilized (33) as described above for Y. pestis CO92.

**Statistical analyses.** Whenever appropriate, one-way analysis of variance (ANOVA) or two-way ANOVA with Tukey’s post hoc test or Student’s t test was employed for data analysis. Kaplan-Meier survival esti-
mates or chi-square analyses were used for animal studies, with \( P \) values of \( \leq 0.05 \) considered significant for all of the statistical tests used.

RESULTS

A high-throughput in vitro screen identified FDA-approved drugs that are effective at inhibiting host cell cytotoxicity during \( Y. \) \( \text{pestis} \) \( \text{CO92} \) infection. Using a modified gentamicin protection assay (37) in conjunction with the MTT assay, we developed a high-throughput screening method to evaluate a library of 780 FDA-approved drugs with known molecular targets for the ability to inhibit the macrophage (murine RAW 264.7) death normally associated with \( Y. \) \( \text{pestis} \) infections (see Fig. S1 in the supplemental material) (38, 39). Of the 780 compounds, we identified a total of 94 drugs that inhibited host cell cytotoxicity for cells infected with WT \( \text{CO92} \), with 36 drugs identified using a pretreatment method and 58 drugs identified using a posttreatment method (see Tables S2 and S3). Drugs were then grouped into either a tier 1 or tier 2 classification, with tier 1 drugs significantly reducing bacterial cytotoxicity to infected macrophages (based on the MTT assay), to the levels seen in uninfected (no bacteria) control macrophages (see Table S2). Tier 2 drugs also significantly reduced the cytotoxicity to infected macrophages, but not to the levels seen for uninfected control macrophages as observed for tier 1 drugs (see Table S3).

Using a posttreatment method, 15 drugs were identified as being tier 1 drugs (Fig. 1), while the other 43 were classified as tier 2 drugs (Fig. 2). Overall, the drugs identified from the pre- and posttreatment screens included antineoplastics, antihistamines, antihypertensives, antivirals, antipsychotics, antidepressants, and hormone receptor modulators, which regulate several molecular pathways in host cells (see Tables S2 and S3 in the supplemental material).

Quantification of bacterial survival in vitro identified 3 drugs capable of limiting host cell death and decreasing intracellular \( Y. \) \( \text{pestis} \) survival. Continuing analysis was limited to tier 1 and tier 2 drugs identified in our posttreatment screen, since these were considered to be the most clinically relevant. Using a bioluminescent \( Y. \) \( \text{pestis} \) \( \text{CO92} \) strain that was created with the \( \text{Tn7-luciferase gene} \) \( (\text{lux}) \)-based system (30, 40, 41), bacterial survival was quantitated in real time. Focusing on the 58 drugs identified from the posttreatment screens and using a gentamicin protection assay with RAW 264.7 macrophages, we tested the effects of the drugs at a concentration of 33 \( \mu \)M on intracellular bacterial survival. From these experiments, 3 of the 58 drugs were shown to affect intracellular bacterial survival: haloperidol, TFP, and pantoprazole (Fig. 3). Haloperidol, an antipsychotic, was classified as a tier 1 drug in our viability screens, while TFP, an antidepressant, and pantoprazole, a treatment for gastroesophageal reflux disease (GERD), were classified as tier 2 drugs (see Tables S2 and S3 in the supplemental material). To eliminate any false-positive results due to host cell cytotoxicity of the 58 drugs, cell viability was assessed by using the MTT assay with the drugs alone (no bacterial challenge) at a concentration of 33 \( \mu \)M for 12 h. Of the 58 drugs tested, none induced any cytotoxicity in RAW 264.7 macrophages.

Three drugs exhibited protection in the murine pneumonic plague infection model. Based on their efficacies from in vitro screens, the 15 drugs classified as tier 1 drugs from our posttreatment viability screens (Fig. 1), as well as the tier 2 drugs TFP and pantoprazole, which both disrupted bacterial intracellular survival (Fig. 2 and 3), were chosen for further analysis in order to determine if the results seen in vitro could be replicated in vivo. Mice were challenged through the i.n. route with WT \( \text{CO92} \) (10 LD\(_{50}\) and, immediately following infection, were dosed with one of the lead drugs through either the oral or i.p. route (see Table S1 in the supplemental material). Dose concentrations were determined either based on the literature (42–50) or empirically (see Table S1). Initially, mice were dosed for up to 6 days at 24-h intervals to mimic the dosing regimen of levofloxacin (18) and were monitored daily for morbidity and mortality. Three drugs, TFP (5 mg/kg), DXP (50 mg/kg), and AXPN (5 mg/kg), were identified to alleviate some early signs of plague disease in mice, such as ruffled fur, lethargy, and inability to groom, although the animals did eventually expire, possibly due to drug toxicity from prolonged treatment, specifically after 4 doses, rather than from the infection. Since the aforementioned three drugs alleviated some symptoms of disease, we sought to optimize the number of doses and drug concentrations to increase animal survival rates (see Table S4).

Through optimization studies, TFP was observed to be equally or even more effective at one dose than at three; therefore, in subsequent mouse pneumonic plague experiments, animals were administered TFP (1.5 mg/kg; one dose), AXPN (3 mg/kg; 3 doses 24 h apart), and DXP (20 mg/kg; 3 doses 24 h apart) by the i.p. route at the time of WT \( \text{CO92} \) infection. To maximize the effectiveness of the chosen drugs, we preferred to deliver the drugs at the time of infection (10 LD\(_{50}\) of WT \( \text{CO92} \)). As noted in Fig. 4A, although the untreated control animals succumbed to infection, 40 to 60% of mice treated with any of these three drugs were protected from developing pneumonic plague, with no clinical signs of the disease or drug toxicity. The remaining 14 drugs evaluated in this model exhibited no survival benefits to animals when administered individually. Although all drugs were given at doses well below the LD\(_{50}\) values (see Table S1 in the supplemental material), additional optimization of dosing regimens may be required to observe beneficial effects in mice.
Because TFP was the most efficacious drug in terms of animal survival after WT CO92 infection, we chose to perform a combinatorial study with levofloxacin to discern if any additive or synergistic effects could be observed. TFP was administered as described above (1.5 mg/kg; one dose given i.p. at the time of infection), while a subinhibitory dose of levofloxacin (0.25 mg/kg) was injected by the i.p. route at 24 h p.i. and subsequently at 24-h intervals for 3 days. While only 20% of mice dosed with 0.25 mg/kg levofloxacin alone were protected from infection, 70% of dually treated mice were protected from developing pneumonic plague (Fig. 4B), indicating that there was some additive effect.

TFP, DXP, and AXPN exhibited a dose-response effect on macrophage viability after Y. pestis CO92 infection. To determine the dose-response effects of the three drugs on macrophage viability following infection, RAW 264.7 macrophages were grown to confluence, infected with Y. pestis at an MOI of 100, and subsequently incubated with TFP, DXP, and AXPN at concentrations of 1, 10, 20, 33, and 50 μM. For all three drugs, the 10, 20, and 33 μM concentrations resulted in statistically significant increases in macrophage viability compared to infected and drug-untreated macrophages (Fig. 4B).
in macrophage viability after WT CO92 infection compared to that of infected, untreated control macrophages (Fig. 5). TFP (Fig. 5A) and AXPN (Fig. 5B) showed the highest levels of macrophage viability at 10 to 20 μM, while DXP (Fig. 5C) demonstrated the highest level of viability at 20 to 33 μM. For all three drugs, macrophage viability began to decrease at 50 μM, with TFP exhibiting the most cytotoxic effect (Fig. 5A).

TFP, DXP, and AXPN exhibited no bactericidal or bacteriostatic effects on Y. pestis CO92. To determine if the three drugs had any bactericidal or bacteriostatic effects, Y. pestis CO92 was grown in the presence of each drug for 24 h at a concentration of 33 μM, as this was the concentration used in the in vitro screening assay and was the highest concentration to exhibit no cytotoxic effects. None of the tested drugs, except for levofloxacin (positive control), negatively influenced the growth of Y. pestis CO92 (Fig. 6A). To confirm that there were no bacteriostatic effects, samples of each drug-containing culture grown for 24 h were reinoculated into fresh HIB and grown for another 24 h. Once again, growth was influenced only by levofloxacin (data not shown). The MICs of these drugs for Y. pestis CO92 were also analyzed up to 100 μg/ml, a concentration well above that used for in vitro screening studies. For all three drugs, the MICs were determined to be above 100 μg/ml (data not shown), indicating that at concentrations distinctly above those that are clinically relevant, no direct antimicrobial effects could be observed for these drugs.

TFP, DXP, and AXPN exhibited no effects on Y. pestis CO92 virulence. Since no bactericidal or bacteriostatic effects of the above-mentioned drugs were observed on Y. pestis CO92, we sought to determine whether any of these drugs could affect bacterial virulence in some way, possibly accounting for the increased mouse survival. We first examined whether the production of T3SS effectors by Y. pestis CO92 was affected by treatment with all three drugs (individually) at 33 μM. After mimicking the conditions of the in vitro screening assay, cell pellets were evaluated for the expression/production of LcrV and YopE specifically by immunoblot analysis. To probe whether Yersinia outer membrane protein (Yop) secretion was affected, supernatants were also evaluated for the presence of YopE by immunoblot analysis. None of

FIG 3 Quantification of intracellular survival of Y. pestis CO92-luc (with the luciferase gene) in RAW 264.7 murine macrophages. RAW 264.7 macrophages were infected with Y. pestis CO92 for 1 h at an MOI of 250. Monolayers were then treated with gentamicin at a concentration of 50 μg/ml for 1 h, washed twice with PBS, and incubated with a maintenance dose of 10 μg/ml gentamicin. At this time, 33 μM drug, or PBS as a control, was added to the monolayers. Following drug treatment, intracellular bacterial survival was measured at 4, 8, and 12 h postinfection through bioluminescence measurement. The data were analyzed by comparison of drug-untreated and infected controls (WT) to treat groups at each time point by using 2-way ANOVA with Tukey’s post hoc test. *, P < 0.01.

FIG 4 (A) Survival analysis of mice infected with Y. pestis CO92 and treated with TFP, AXPN, DXP, or levofloxacin. Mice were challenged by the intranasal (i.n.) route with 10 LD50 (1 LD50 = 500 CFU) of WT Y. pestis CO92 and administered TFP (n = 15; pooled from three independent experiments) at a dose of 1.5 mg/kg, AXPN (n = 10; pooled from two independent experiments) at a dose of 3 mg/kg, DXP (n = 15; pooled from three independent experiments) at a dose of 20 mg/kg, or saline (n = 20; pooled from four independent experiments) as a control by the intraperitoneal (i.p.) route at the time of infection. Levofloxacin was administered at a dose of 5 mg/kg at 24 h p.i. (n = 10; pooled from two independent experiments). Animals were dosed for 3 days (24-h intervals) for levofloxacin, DXP, and AXPN or given only 1 dose of TFP at the time of bacterial infection and were observed for mortality for 21 days. The data were analyzed for significance by use of Kaplan-Meier survival estimates. The P-values were determined based on comparison of the results for each of the drug treatment groups to the results for saline-treated and infected controls (WT) (P = 0.0374 for AXPN, P = 0.0044 for DXP, P = 0.0007 for TFP, and P = 0.0003 for levofloxacin). (B) Survival analysis of mice infected with Y. pestis CO92 and treated with a combination of TFP and a subinhibitory dose of levofloxacin. Mice were challenged by the i.n. route with 10 LD50 of WT Y. pestis CO92, and two groups were administered TFP at a dose of 1.5 mg/kg by the i.p. route at the time of infection. A control group of animals was given saline at the time of infection. As described in the text, at 24 h p.i., levofloxacin at a dose of 0.25 mg/kg was administered i.p. to animals. Groups receiving TFP, levofloxacin, or saline alone served as controls. The data were analyzed for significance by use of Kaplan-Meier survival estimates. The P-values were determined based on comparison of the results for each of the drug treatment groups to the results for saline-treated and infected controls (WT). P = 0.0006 for TFP, P = 0.0190 for levofloxacin, and P < 0.0001 for TFP and levofloxacin.
the tested drugs affected the expression/production of LcrV and YopE in cell pellets or secretion of YopE into culture supernatants (Fig. 6B). We also assessed whether the Pla protease activity of Y. pestis CO92 was affected by the presence of the three drugs individually. As with the evaluation of T3SS components, the conditions of the in vitro assay were mimicked, with bacteria grown in the presence of all three drugs (individually) at 33 μM. Protease activity was then measured using a fluorometric assay with the Pla substrate (34). As with the T3SS effectors, we observed no drug inhibition or impact on Pla protease activity (Fig. 6C). Taken together, the results showed that the three antiplague candidate drugs identified through in vitro and in vivo screens reduced bacterial pathogenesis seemingly by targeting host cell pathways rather than the bacterial pathogen directly.

TFP exhibited protection in a murine model of S. Typhimurium infection, with no direct bactericidal or bacteriostatic effects. Since TFP exhibited the most promising results in our Y. pestis infection model, we decided to examine if protection could be afforded to other bacterial infection models. As TFP has previously been shown to increase survival in a septicemic mouse model of Salmonella infection (35), we attempted to corroborate these findings by using a different strain of S. Typhimurium and a higher bacterial challenge dose. Mice were dosed once with 1.5 mg/kg TFP i.p. 3 h before infection, as this was the most successful dosing regimen reported (35), and then subsequently challenged by the i.p. route with 1.0 × 10⁶ CFU (1,000 LD₅₀) of bacteria. Through this model, a statistically significant increase in animal survival was observed for TFP-treated animals (60% survival) compared to untreated controls (0% survival) or levofloxacin-treated controls (0% survival for 5-mg/kg and 0.25-mg/kg dosing) (Fig. 7A).

To evaluate if TFP had any bactericidal or bacteriostatic effect on this pathogen, S. Typhimurium 14028 was grown in the presence of 33 μM TFP in order to mimic the concentration used for Y. pestis CO92. At this concentration, TFP was observed to have no effect on bacterial growth (Fig. 7B). In order to determine if there were any bacteriostatic effects, samples grown for 24 h in drug-containing medium were reinoculated into fresh LB broth and grown for another 24 h. As observed with Y. pestis, the growth of S. Typhimurium was not affected by TFP treatment. The MIC of TFP for S. Typhimurium 14028 was also analyzed up to 100 μg/ml, as the reported MIC for strains NCTC 11 and NCTC 74 is 80 μg/ml (35), and it was determined to be above 100 μg/ml (data not shown), indicating that TFP was not bactericidal to S. Typhimurium 14028 at clinically relevant concentrations.

TFP exhibited protection in a murine model of C. difficile infection. To determine whether TFP possessed antibacterial properties beyond Y. pestis and S. Typhimurium 14028, we chose to test the efficacy of TFP in a C. difficile infection model. Following the parameters of our S. Typhimurium infection model, mice were dosed with 1.5 mg/kg TFP by the i.p. route 3 h before infection and subsequently challenged by oral gavage with 10⁹ C. difficile (VPI 10463) spores. Although the difference was not statistically significant, we did observe an increase in survival of TFP-treated animals compared to vancomycin-treated animals. We did, however, observe a significant increase in survival (60%) for TFP-treated animals compared to the untreated controls, which all succumbed to infection (Fig. 7A). Following an initial decrease in body weight, TFP treatment resulted in recovery of animal body weights to weights that were significantly higher.
than those of vancomycin-treated animals (Fig. 8B). Furthermore, histological examination of tissues at necropsy revealed significant differences between untreated and TFP-treated animals. Untreated animals demonstrated significant signs of inflammation, disruption of normal tissue architecture, and edema that were not apparent in TFP-treated mice (Fig. 8C).

**DISCUSSION**

In efforts to identify novel antibacterial chemotherapeutics from a library of 780 FDA-approved drugs, we employed a high-throughput screening method based on evaluation of *Y. pestis*-induced macrophage cytotoxicity. During infection, *Y. pestis* preferentially targets host macrophages, as the organism is protected from contact with other immune components and acquires the ability to evade subsequent phagocytosis (23, 24). Macrophages are key effectors of the host innate immune response to infection and intracellular survival and growth of *Y. pestis*, and since macrophages play a pivotal role in the pathogenesis of the plague (23, 24, 51), this *in vitro* model was well suited for our screening purposes. In total, 94 FDA-approved drugs were identified in our screen as being able to inhibit *Y. pestis*-induced macrophage cytotoxicity. Candidate drugs were placed into tier 1 and tier 2 categories based on the degree to which treatment reduced *Y. pestis*-induced macrophage cytotoxicity (see Tables S2 and S3 in the supplemental material).

The decreased host cell cytotoxicity associated with *Y. pestis* infection observed for the drugs could have been a result of a reduction in intracellular survival of the invading pathogen or the stabilization of infected macrophages (e.g., a disruption of the apoptotic pathway). Some drugs identified in *in vitro* screens have previously been shown to have some antimicrobial activity, such as TFP and proton pump inhibitors, such as pantoprazole, which are known antituberculars (52, 53). After validating that none of the 58 drugs from the more clinically relevant posttreatment
screens (see Tables S2 and S3 in the supplemental material) had any cytotoxic effects on macrophages alone (data not shown), three identified drugs that inhibited host cell death were also shown to decrease bacterial intracellular survival (Fig. 3). One drug, haloperidol, was classified as a tier 1 drug, while TFP and pantoprazole were classified as tier 2 drugs. As the remaining 55 compounds did not decrease intracellular bacterial survival but did exhibit increases in macrophage viability, we speculate that these drugs stabilize macrophages to prevent their death associated with infection. This, in turn, may aid in altering subsequent immune responses to the infection to better allow for bacterial clearance at a later time point in the whole animal. Indeed, it has been shown that early inhibition of macrophage apoptosis following *Y. pestis* infection aids in reducing inflammation and allowing for better bacterial clearance (51).

From the *in vitro* studies, a set of 17 drugs, including 15 tier 1 drugs and 2 tier 2 drugs (TFP and pantoprazole) (Fig. 1 and 2), were prioritized for evaluation to determine if efficacy *in vitro* could be translated *in vivo*. Our results indicated that three drugs, AXPN (tier 1), DXP (tier 1), and TFP (tier 2), significantly increased the survival of mice in a pneumonic plague model (Fig. 4A). Additional studies with TFP indicated that combinatorial treatment with TFP and a subinhibitory dose of levofloxacin showed an additive effect on the animal survival rate (Fig. 4B). The poor efficacies of the other 14 drugs, particularly haloperidol and pantoprazole, which were both able to inhibit the intracellular survival of *Y. pestis* CO92, may indicate the need for additional optimization of *in vivo* dosing regimens, as was needed for our three reported lead drugs (see Tables S1 and S4 in the supplemental material).

In order to evaluate if there was a dose–response effect on macrophage viability from TFP, AXPN, or DXP treatment following infection with *Y. pestis* CO92, we employed the same procedure used for the initial high-throughput screen. Using drug concentrations of 1, 10, 20, 33, and 50 μM, it was observed that there was a dose-response effect for each drug (Fig. 5), and at the highest concentration (50 μM), signs of cytotoxicity could be observed, particularly for TFP (Fig. 5A). These results validated the use of 33 μM drug for initial screening procedures, as high efficacy values were observed at this concentration, while the drugs also remained noncytotoxic to macrophages.

In order to elucidate whether *Y. pestis* was being affected directly, AXPN, DXP, and TFP were evaluated for possession of either bactericidal or bacteriostatic activity. Our results indicated that none of the drugs had a direct bactericidal/bacteriostatic effect (Fig. 6A), suggesting that these drugs may inhibit bacterial virulence factors (not associated with bacterial growth) or may prevent plague pathogenesis through host-directed targets. To assess if these drugs were acting through bacterial or host targets, we evaluated the expression of two T3SS effector proteins, LcrV and YopE, the latter of which destroys actin monofilaments. No measurable differences in the production levels of these antigens were observed in cell pellets following drug treatments, and likewise, no differences in the secretion levels of YopE were observed in culture supernatants (Fig. 6B). Furthermore, no measurable differences in protease activity of Pla, another key *Y. pestis* virulence factor, were observed when the bacterium was grown in the presence of each drug individually (Fig. 6C). These data provided further evidence that these drugs were likely operating through host-directed targets to alleviate disease pathogenesis.

To evaluate if these drugs had broad applicability, we tested the therapeutic efficacy of TFP in mouse models of *S. Typhimurium* and *C. difficile* infections, since TFP gave the highest survival rates for mice infected with *Y. pestis*. TFP significantly increased survival in a *C. difficile* infection model (Fig. 8A), and likewise, the drug increased animal survival in an *S. Typhimurium* infection model (Fig. 7A), confirming the previously reported results (35). Interestingly, similar to the results observed with *Y. pestis*, TFP exhibited no direct bactericidal or bacteriostatic effects on *S. Typhimurium* (Fig. 7B), further indicating that the broadly acting drug TFP likely targets common host cell pathways exploited by multiple bacterial pathogens during infection.

The use of host-directed agents for the treatment of antibiotic-resistant bacteria has recently garnered much attention (5, 54, 55). Additionally, the repurposing of FDA-approved drugs offers the advantage of readily moving *in vitro* candidates to *in vivo* models, since the LD50 and 50% effective dose (ED50) values are readily available and considerable information about their molecular targets and host pathway interactions is known.

TFP belongs to a class of phenothiazine drugs and is a dopamine antagonist presently used as an antipsychotic for patients...
with psychotic disorders, such as schizophrenia and anxiety, and it can also be used to treat nausea caused by chemotherapy (56). The current therapeutic dose for TFP varies based on the condition it is being used to treat, but it is generally recommended to be no more than 40 mg a day for schizophrenia and no more than 6 mg for nonpsychotic anxiety (56). However, although TFP can be prescribed for long-term human use, we administered this drug (1.5 mg/kg dose) only once in mice. In addition to its known therapeutic uses, TFP has been reported to protect human lung fibroblasts from intoxication caused by\textit{C. difficile} toxin B (57) and to reduce \textit{S. Typhimurium} virulence in both \textit{in vitro} and \textit{in vivo} models. TFP is a known antimycobacterial, has been shown to have bactericidal properties against several pathogens, including staphylococci, vibrios, \textit{Salmonella}, and \textit{Pseudomonas} spp. (35, 52, 58), and is known to accumulate in macrophages, enhancing its activity against intracellular pathogens. However, the bactericidal activity of TFP varies based on bacterial strain for various pathogens (35, 52, 59), which may account for why we observed no bactericidal effects for \textit{S. Typhimurium} strain 14028 or \textit{Y. pestis} (Fig. 6B and 7B). Besides TFP having direct bactericidal effects, a decrease in intracellular survival of \textit{S. Typhimurium} strain SL1344 in epithelial cells treated with TFP at 10 \(\mu\)g/ml was described to be the result of host autophagy modulation activities of TFP (60). Since no direct bactericidal effects were observed in \textit{in vitro} models of \textit{Y. pestis} and \textit{S. Typhimurium} infections, TFP may target the autophagy pathway in macrophages to promote bacterial clearance.

AXPN is a tricyclic antidepressant drug which inhibits the uptake of norepinephrine and serotonin and blocks dopamine’s effect on dopamine receptors. The current therapeutic dosage of AXPN is recommended not to exceed 600 mg daily, with the average dosage being around 300 mg daily, placing the dosage of 3 mg/kg used in this study well below that of the therapeutic window (61). In addition to its use as an antidepressant, AXPN and its metabolites have shown efficacy for alleviating cancer drug toxicity and resistance through potent bacterial \(\beta\)-glucuronidase and \textit{P-glycoprotein} transporter inhibitor activities (62–64). In regard to host responses to infection, AXPN has been shown to have membrane-stabilizing activity, which causes inhibition of a fast inward passive Na\textsuperscript{+} current, resulting in membrane hyperpolarization and upregulation of immune cell activity (65–67). Though AXPN was not observed to decrease bacterial intracellular survival in macrophages, its described membrane-stabilizing activity may be responsible for the increase in host cell and animal survival seen in \textit{Y. pestis} infection models (Fig. 1 and 4A). A reduction in immune cell cytotoxicity caused by AXPN may aid in overall clearance of the pathogen through an altered host inflammatory response and promotion of phagocytosis, which is normally inhibited during later stages of \textit{Y. pestis} infection (38, 51).

Unlike TFP and AXPN, which are both psychoactive drugs, DXP is a breathing stimulant that causes an increase in tidal volume and respiratory rate through stimulation of chemoreceptors in the carotid bodies independent of oxygen levels (68, 69). Similar to the case for TFP and AXPN, the therapeutic dose of DXP...
survival following treatment with the drugs TFP, AXPN, and DXP to better gauge their therapeutic potential in the delayed-onset treatment to improve viability and optimization in delayed-onset treatment to better gauge their therapeutic potential in the future. Further, whether these drugs’ mechanisms of action against *Y. pestis* and other bacterial pathogens are modulated at the level of the bacteria or the host also needs to be delineated. Finally, whether these drugs act through similar or different mechanisms in each model of infection and whether these drugs have applicability as therapeutics against other pathogens, specifically multiple-antibiotic-resistant ones, remains to be elucidated.

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