Repurposing Clinically Approved Drugs for the Treatment of Bacillus cereus, a Surrogate for Bacillus anthracis

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ABSTRACT: Of the numerous infectious diseases afflicting humans, anthrax disease, caused by Bacillus anthracis, poses a major threat in its virulence and lack of effective treatment. The currently lacking standards of care, as well as the lengthy drug approval process, demonstrate the pressing demand for treatment for B. anthracis infections. The present study screened 1586 clinically approved drugs in an attempt to identify repurposable compounds against B. cereus, a relative strain that shares many physical and genetic characteristics with B. anthracis. Our study yielded five drugs that successfully inhibited B. cereus growth: dichlorophen, oxiconazole, sulcotidil, bithionol, and hexestrol. These drugs exhibited varying levels of efficacy in broad-spectrum experiments against several Gram-positive and Gram-negative bacterial strains, with hexestrol showing the greatest inhibition across all tested strains. Through tests for the efficacy of each drug on B. cereus, bithionol was the single most potent compound on both solid and liquid media and exhibited even greater eradication of B. cereus in combination with sulcotidil on solid agar. This multifaceted in vitro study of approved drugs demonstrates the potential to repurpose these drugs as treatments for anthrax disease in a time-efficient manner to address a global health need.

INTRODUCTION

Infectious diseases have long posed a threat to human health and have been an ongoing motivation for drug research and development. In addition to the difficulty of developing enough treatments for the multitude of pathogenic diseases, there is the challenge of matching the pace of drug development with the rapid speed at which pathogens develop resistance to existing treatments.

Among these pathogens is the bacterium Bacillus cereus, a Gram-positive, aerobic, and motile species of the phylum Firmicutes. B. cereus is commonly found on livestock as well as decaying plant matter or soil, allowing it many avenues to enter humannmade food processing areas. Its resilience to a broad range of environments poses a challenge for facilities and physicians alike attempting to eliminate this pathogen. Like its Firmicutes counterparts, B. cereus forms endospores that are resistant to desiccation, extreme temperatures, and radiation and allow the bacterium to remain dormant until more favorable conditions arise. In addition, the hydrophobicity of its endospores allows the bacterium to effectively adhere to a variety of both biotic and abiotic surfaces, indicating that humans are not excluded from its options for settlement.

Upon ingestion of this pathogen, usually from food sources, B. cereus causes gastrointestinal tract infections in humans, which leads to food poisoning symptoms such as diarrhea, nausea, and vomiting. Aside from food-borne infections, B. cereus can also infect an organism via inhalation of spores or infections from open wounds.

B. cereus is a widely used and relatively safer surrogate for the more critical pathogen, Bacillus anthracis, the causative bacterium of anthrax. While the two Bacillus species exhibit very similar physical characteristics, are close phylogenetic relatives, and share homologous plasmid structures, they greatly differ in their virulence. Although cutaneous forms of anthrax are treatable with antibiotics and are often nonfatal, anthrax has multiple mechanisms of infection that make it an imminent threat to humans. Respiratory infection by B. anthracis has the highest fatality rate of up to 90%. Its nonspecific symptoms such as fever and cough make it difficult to diagnose upon infection and can lead to dyspnea, hypothermia, cardiac and pulmonary shock, and almost inevitable death despite antibiotic treatment. In addition to cutaneous and respiratory infections, anthrax also takes form in gastrointestinal tract infections, as well as a more recently identified form of injectional anthrax, which has a mortality rate of over 33%.

While immunoglobulin antitoxins, corticosteroids, and other toxin inhibitors are current areas of research as potential treatments to be used in conjunction with antibiotics, there still...
remains a lack of one effective treatment against anthrax that ensures survival.\textsuperscript{13,14,18} This lack of decisive treatment against anthrax, the recent emergence of a new pathway of infection, and the remarkably few antitoxin drugs currently approved for anthrax treatment indicate the need for further discovery of an antibacterial treatment.\textsuperscript{14} Furthermore, our current availability of antitoxins would not suffice to treat patients in the case of a large-scale anthrax incident.\textsuperscript{18,19} A novel treatment for anthrax would not only pose extremely valuable for the clinical treatment of infected individuals but also serve as a crucial measure to ensure the health and safety of large-scale populations.

In consideration of ongoing research efforts in search of an effective treatment for anthrax, we searched for novel uses for approved drugs as a safe and relatively faster method of identifying possible treatments for bacilli infections. Our research group screened clinically approved drugs and their effects on \textit{B. cereus}, which in the future could further be tested for their antianthrax efficacy.

\section*{RESULTS AND DISCUSSION}

\textbf{Drug Screenings.} To evaluate a broad spectrum of currently approved drugs to potentially repurpose, we used Johns Hopkins Clinical Compound Library (JHCLL) version 1.33, which consisted of 1586 U.S. FDA-approved drugs, foreign-approved drugs, and drugs undergoing phase 2 or 3 clinical trials. All drugs were dissolved in water or dimethyl sulfoxide (DMSO) at a concentration of 3.3 mM and arrayed

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Preliminary screening criteria for hits prioritization. Hits were determined based on significantly larger and darker areas and intensities of zones of inhibition compared to DMSO, the negative control. Rifampin was referenced as a positive control. One microliter of each 3.3 mM drug stock was spotted immediately following spreading 100 \textmu L of \textit{B. cereus} on solid lysogeny broth (LB) media and then incubated at 37 \textdegree C overnight. All images were scaled to 15 mm. Numbers quantify the depth of inhibition as a multiple of each hit’s pixel count relative to DMSO.\textsuperscript{51}}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Drug Name} & \textbf{Structure} & \textbf{Indication} \\
\hline
Dichlorophen & & Anthelmintic \\
\hline
Sulocidil & & Vasodilator \\
\hline
Oxiconazole & & Antifungal \\
\hline
Bithionol & & Anthelmintic \\
\hline
Hexestrol & & Nonsteroidal estrogen replacement \\
\hline
\end{tabular}
\caption{Summary of Drug Hits Effective against \textit{B. cereus}\textsuperscript{a}}
\end{table}

\textsuperscript{a}Names, structures, and indications of drug hits identified in drug screen on solid media against \textit{B. cereus}.
in 27 96-well plates.\textsuperscript{20} Columns 1 and 12 of each plate contained only DMSO and served as negative controls. Rifampin (rifampicin) from the JHCCL served as our positive control for screenings on solid media.

Compounds that were further screened, hereafter referred to as hits, were purchased separately and included dichlorophen (Alfa Aesar; Ward Hill, MA), oxiconazole nitrate (Sigma-Aldrich; St. Louis, MO), sulcotidil (Sigma-Aldrich), bithionol (Sigma-Aldrich), and hexestrol (Alfa Aesar). Hits were purchased as powders and prepared in DMSO to a stock concentration of 3.3 mM. All drugs were stored at $-20^\circ$C and thawed at room temperature prior to use.

For all tests with the exception of tests varying the bacterial cell concentrations (refer to MIC tests), 100 $\mu$L of the liquid media containing $B.\ ceru$ was spread onto each plate ($d = 145$ mm), containing approximately $3.34 \times 10^7$ cells (McFarland's scale, optical density (OD) at 600 nm).\textsuperscript{21}

**Hit Prioritization from Preliminary Drug Screenings.**

The initial preliminary screening of 1586 drugs in the JHCCL library yielded 227 drugs that exhibited inhibitory effects on $B.\ ceru$, identified by having zones of inhibition larger than DMSO. From these, known antibacterials, as well as drugs having reported immunosuppressive qualities, were eliminated, resulting in 65 drugs of interest for our next confirmatory screens. Rifampin was used as a positive control. Two repeated screens allowed us to isolate five hits that had consistent zones of inhibition significantly larger than DMSO, which included dichlorophen, oxiconazole, sulcotidil, bithionol, and hexestrol (Figure 1 and Table 1).

**MIC Identification of Hits in Solid and Liquid Screenings.** Minimum inhibitory concentrations (MICs) on solid media were defined as the lowest concentration at which 1 $\mu$L of a hit produced a zone of inhibition larger than DMSO (Table 2). On a lawn of 100 $\mu$L of $B.\ ceru$ ($3.34 \times 10^7$ cells/Petri dish), bithionol exhibited the lowest minimum inhibitory concentration of 0.013 mM. MICs of oxiconazole, dichlorophen, and sulcotidil were found to be 0.41 mM. Hexestrol's MIC was the highest concentration of the hits at 0.83 mM. Bithionol and dichlorophen exhibited slightly decreased inhibitory effects on 108.3 $\mu$L of $B.\ ceru$ with MICs of 0.025 and 0.83 mM, respectively, but maintained those MICs at the highest volume of $B.\ ceru$ at 116.7 $\mu$L. The MIC of oxiconazole remained constant from 100 to 108.3 $\mu$L of $B.\ ceru$ ($3.62 \times 10^7$ cells) but exhibited a slight decrease in its MIC of 0.41–1.65 mM on 116.7 $\mu$L of $B.\ ceru$ ($3.90 \times 10^7$ cells). Sulcotidil and hexestrol had constant MICs for all three volumes of $B.\ ceru$ lawns.

MICs in liquid were defined as the lowest concentration, at which the drug producing a bacterial growth curve significantly inhibited compared to the negative control (Figures 2 and 3). These were found to be, in ascending order: bithionol at 0.13 $\mu$M, oxiconazole at 4.13 $\mu$M, dichlorophen at 6.00 $\mu$M, hexestrol at 16.50 $\mu$M, and sulcotidil at 20.30 $\mu$M (Table 3), with their 50% effective concentrations (EC\textsubscript{50}) listed in Table 4. MICs were identified at dilutions of the highest precision that significantly inhibited bacterial growth in a consistent manner.

**Synergistic Efficacy of Sub-MIC Combinations in Solid and Liquid Screenings.** Drugs were combined at sub-MICs, or the highest concentration prior to MIC at which each drug did not significantly inhibit bacterial growth, to assess for synergistic effects of drugs at lower concentrations. Sub-MICs on solid media were found at 1 spotted $\mu$L of 0.0065 mM for bithionol; 0.052 mm for oxiconazole, sulcotidil, and dichlorophen; and 0.41 mm for hexestrol. Combinations that showed efficacy on solid media, followed by their increase relative to the average of their individual sub-MIC drugs (calculated as the mean hit intensity (MHI) of drug combinations/MHI of individual drugs), were as follows: bithionol and oxiconazole, 8.22-fold; hexestrol and bithionol, 6.06-fold; and bithionol and sulcotidil, 8.53-fold (Figure 4d). The following pairs did not have significant efficacy as combinations on solid media: oxiconazole and hexestrol; oxiconazole and sulcotidil; sulcotidil and hexestrol; dichlorophen and sulcotidil; dichlorophen and oxiconazole; and hexestrol and dichlorophen (Figure S1). Dichlorophen and bithionol were not tested as a combination due to the structure–activity relationship predicting similar mechanisms of action.\textsuperscript{22,23}

The use of sulcotidil was halted in 1985 worldwide following reports of sulcotidil-induced liver toxicity. In hopes of lowering future toxicity, further dilutions of sulcotidil were tested on solid media in combination with bithionol held at a constant concentration of 0.0065 mM. Inhibitory effects of sulcotidil and bithionol as folds relative to the average of their individual sub-MICs at listed sulcotidil concentrations (calculated as the MHI of drug combinations/MHI of individual drugs) were 0.026 mM, 6.88-fold; 0.013 mM, 4.37-fold; 0.0064 mM, 14.73-fold; and 0.0032 mM, 5.32-fold (Figure 5).

Sub-MICs in the liquid assays were found to be 0.03 $\mu$M for bithionol, 1.00 $\mu$M for oxiconazole ($p = 0.3720$), and 0.013 $\mu$M for dichlorophen ($p = 0.8215$), and 16.50 $\mu$M for sulcotidil (Table 3). All of these concentrations are near but lower than the predicted EC\textsubscript{50} for these drugs (Table 4). In combination with liquid media, efficacious combinations and their percent inhibition were dichlorophen and sulcotidil, 99.44% (Figures 6a and S2a); oxiconazole and sulcotidil, 90.09% (Figures 6b and S2b); oxiconazole and bithionol, 98.53% (Figures 6c and S2c); sulcotidil and bithionol, 98.12% (Figures 6d and S2d); hexestrol and bithionol, 95.20% (Figures 6e and S2e); and hexestrol and sulcotidil, 93.07% (Figures 6f and S2f). Combinations that did not exhibit synergistic efficacy in liquid media were the following: oxiconazole and hexestrol, oxiconazole and dichlorophen, and hexestrol and dichlorophen (Figure S3).

**Broad-Spectrum Efficacy of Hits.** MIC on solid media was identified for 1 $\mu$L of each hit on eight unique bacterial strains (Table 5). Dichlorophen inhibited *Micrococcus luteus* until an MIC of 1 $\mu$L of 0.41 mM, equal to its MIC found on $B.\ ceru$. However, it showed higher MIC concentrations of 1

![Table 2. MIC of Hits on Solid LB Media at Varying Concentrations of $B.\ ceru$](https://pubs.acs.org/acsomega/2020/5/21939/acsomega.0c03207.xml#table2)
μL on Bacillus subtilis at 1.65 mM, Citrobacter freundii at 0.83 mM, Serratia liquefaciens at 0.83 mM, Vibrio natriegens at 0.83 mM, Acinetobacter calcoaceticus at 1.65 mM, and Escherichia coli at 1.65 mM. It did not inhibit Enterobacter aerogenes growth in a significant manner. Compared to B. cereus inhibition, oxiconazole inhibited M. luteus and B. subtilis until an equal or lower MIC of 1 μL of 0.063 mM and 0.41 mM, respectively, but did not exhibit inhibition of any other bacterial strain tested. Suloctidil inhibited M. luteus until an improved MIC relative to B. cereus of 0.21 mM. MICs were found at higher concentrations of 1 μL of 3.30 mM, B. subtilis at 1.65 mM, C. freundii at 0.83 mM, A. calcoaceticus at 0.83 mM, and E. coli at 3.30 mM and did not show inhibitory effects on V. natriegens. Bithionol inhibited B. subtilis and A. calcoaceticus until an MIC of 1 μL of 0.013 mM, a concentration equal to the MIC on B. cereus. It showed MICs at a higher concentration on M. luteus at 1 μL of 0.025 mM, E. aerogenes at 1.65 mM, C. freundii at 0.025 mM, S. liquefaciens at 0.21 mM, and V. natriegens at 3.30 mM. Hexestrol had improved MICs relative to B. cereus on M. luteus at 1 μL of 0.21 mM, B. subtilis at 0.83 mM, C. freundii at 0.41 mM, and V. natriegens at 0.41 mM. It did not inhibit E. aerogenes, S. liquefaciens, A. calcoaceticus, and E. coli.

With the exception of suloctidil, all of the hits we discovered in our assay contain two phenols (Figure 1). It was recently shown that bithionol has highly potent activity against methicillin-resistant Staphylococcus aureus (MRSA).24,25 Bithionol was shown to act on bacterial membranes by attaching to the phosphate heads on the bacterial membrane via its polar group and by embedding in the membrane and disrupting it using its hydrophobic groups. However, the same studies observed that the antibiotic efficacy of bithionol is not observed against Gram-negative bacteria. Similar observations were made by other recent studies for other bisphenolic chemicals, showing that they inhibit bacteria by targeting the bacterial membrane and demonstrating additive or synergistic efficacy with other antibiotics.26−29 These observations were generally confirmed by our study: all five hits were effective against all Gram-positive bacteria, but none of the hits were effective against all Gram-negative bacteria (Table 5). Moreover, all five hits were, in general, less effective against Gram-negative than against Gram-positive bacteria, with higher MIC values for Gram-negative microbes. Thus, we hypothesize that our bisphenolic hits, namely dichlorophen, oxiconazole, and hexestrol, act by the membrane-targeting mechanism similar to that shown by bithionol.

We also hypothesize that suloctidil also binds to and disrupts bacterial membranes, although possibly by another mechanism. Suloctidil contains a fat-soluble saturated eight-carbon-long tail, which resembles the structure of detergents (Table 1).
Detergent compounds consist of a hydrophobic polycarbon tail and a hydrophilic charged headgroup. When dissolved in water at a given concentration, detergent molecules will form micelles. Micelle structures include the hydrophobic tail in the interior and the charged headgroup at the exterior of the micelle. The minimal concentration at which micelles are formed in a solution is called the “critical micelle concentration” (CMC). It has been shown that at or above the CMC, detergent micelles bind to and denature most proteins, where the hydrophobic core of the micelle binds to the hydrophobic regions of proteins. It is also known that at submicellar concentrations, detergents may bind to specific proteins. Therefore, since detergent-like suloctidil displayed high potency on solid media and low potency in liquid media, it is possible that it acts against a bacterial membrane by a micelle-free mechanism in solid media at a lower concentration and by a micelle-dependent mechanism in liquid culture at higher concentrations.

The high risk of fatality within days of infection by respiratory and gastrointestinal anthrax demonstrates a pressing need for an effective treatment for anthrax disease. In addition to the serious virulence of the disease, there are currently shortages of existing treatments, namely, penicillin, one of the primary treatments for the infection often used as a first-line treatment. Given these needs, we assessed 1586 clinically approved drugs in the Johns Hopkins Clinical Compound Library to assess opportunities to repurpose existing drugs as treatments for infection by B. cereus, a surrogate for B. anthracis.

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Table 3. MIC of Hits in Liquid LB Media

| compound     | MIC in liquid (μM) | sub-MIC in liquid (μM) |
|--------------|--------------------|------------------------|
| dichlorophen | 6.00               | 4.13                   |
| oxiconazole  | 4.13               | 1.03                   |
| suloctidil   | 20.30              | 16.50                  |
| bithionol    | 0.13               | 0.065                  |
| hexestrol    | 16.50              | 8.30                   |

“MICs were defined at the lowest concentration of each hit that consistently inhibited bacterial growth in a significant manner relative to the negative control, untreated B. cereus in liquid LB media.

Table 4. Predicted EC50 of Hits

| compound     | EC50 (μM) |
|--------------|-----------|
| dichlorophen | 6.63      |
| oxiconazole  | 3.59      |
| suloctidil   | 17.84     |
| bithionol    | 0.089     |
| hexestrol    | 8.77      |

“Half-maximal effective concentrations were calculated using polynomial trendlines on Excel (R² > 0.98).
cytotoxicity of our hits at 16 μM observed in our liquid tests. Bithionol was replaced by another drug with better antihelmintic efficacy, praziquantel. In addition, bithionol was used as an antibacterial in cosmetic products before being discontinued in 1967 due to its photosensitizing effects. Bithionol has been shown to reach serum at 225–480 μM after the oral administration of 50 mg/kg, which is significantly higher than the MIC of 0.13 μM observed in our liquid tests. Bithionol was tested in combination with all hits except its structural analogue, dichlophen. While the two compounds have highly similar structures, dichlophen had considerably lower inhibition of B. cereus growth on solid and in liquid media in dilutions below 16 μM. Despite this lower potency, its current use as an oral drug makes it a valuable hit with the potential to be repurposed as an internal treatment for B. cereus and likely B. anthracis treatment. Both bithionol and dichlophen share structures with two free phenolic hydroxyl groups, allowing them to be readily conjugated and absorbed in the gastrointestinal tract, as shown in dichlophen administration to rats. While the pharmacokinetics of dichlophen has not been fully determined, in the context of repurposing, dichlophen’s clinical use as an oral anticestodal, antifungal, and antimicrobial treatment suggests its potential as a treatment for inhalatory, gastrointestinal, or injection anthrax infection.

Suloctidil’s extremely high synergistic efficacy of almost 100% B. cereus growth inhibition independently and in all combinations with other hits at its sub-MIC in liquid assays suggests a possibility for inhibitory efficacy at further dilutions in combination therapies (Figure 5). In the present study, the concentration of suloctidil tested in highly efficacious combinations in liquid (16 μM) was twice as concentrated as previously reported maximal plasma concentrations and equal to reported cytotoxic levels in RAW264.7 mouse macrophage cells and C32 human melanoma cells, warranting further testing of lower concentrations of suloctidil in combination with other hits. While suloctidil’s hepatotoxic effects caused its removal from the market from its original indication of vasodilation to treat arterial diseases, its potential to treat a dangerously lethal infection such as anthrax and its possibility of efficacy at lower concentrations than previously indicated suggest a potential future against B. anthracis.

Despite its previous indication as a vasodilator, suloctidil’s tremendous efficacy in combinations on solid media cannot be ignored. Its constant MIC in all tested concentrations of B. cereus cells spread on solid media suggests its consistent efficacy in later stages of infection in the context of topical administration. While tests of suloctidil alone on solid media exhibited MICs at relatively higher concentrations, combinations with drugs such as bithionol showed tremendous synergistic effects, with clear inhibition of 3.14-fold until the lowest tested concentration of 3.22 μM (Figure 5). While previous indications required oral or intravenous adminis-
tration of sulocitidil and therefore would require these routes of administration in the context of repurposing, this high efficacy of sulocitidil at extremely low concentrations in combination treatments on solid agar suggests a promising future of sulocitidil in not only internal but also topical treatment worth pursuing with further study.46−48

Oxiconazole, currently used as a topical antifungal cream, has demonstrated an absorption of 16.2 μM in the epidermis and 1.29 μM in the deeper corium.49 Due to the incongruent translation of in vitro agar MIC data to epithelial absorption, the reported MICs on solid agar do not conclusively state whether oxiconazole inhibits B. cereus at a concentration lower than reported values. However, oxiconazole’s shown synergistic efficacy with sulocitidil and bithionol on solid media at combinations at its sub-MIC suggests its enhancing effects of other treatments when combined on solid agar, suggesting the promising potential of its use in combination therapy and requiring future studies in the human epithelium. Additionally, the clear inhibitory effects in liquid media at a lower concentration than absorbed in the epidermis (10 μM) suggest the possibility of efficacy in treating B. cereus and, therefore, B. anthracis at concentrations lower than currently indicated (Figure 2b). Despite its marked cytotoxicity in RAW264.7 mouse macrophage cells at 16 μM, the concentrations tested in the present study were lower than previously studied and suggest efficacy at concentrations below toxic levels.30,32,33

While hexestrol had lower potency than its antifungal counterpart, oxiconazole, it has been shown to have lower toxicity at 16 μM. Due to its current indication as an injected and orally administered estrogen, its synergistic efficacy in liquid media in combination with bithionol and sulocitidil reveals the potential for use in combination with these drugs within its current route of administration.50

Figure 6. Synergistic efficacy of combinations in liquid assay. (a−f) show increased efficacy of combinations compared to individual sub-MIC efficacy. Pairings in liquid media that exhibited efficacy in combination were (a) dichlorophen (DCL, p = 0.0171) and sulocitidil (SDL, p = 0.9931), (b) oxiconazole (OXI, p = 0.0510) and sulocitidil (SDL, p = 0.1954), (c) oxiconazole (OXI, p = 0.3153) and bithionol (BTN, p = 0.1898), (d) bithionol (p = 0.1913) and sulocitidil (p = 0.2139), (e) hexestrol and bithionol, and (f) hexestrol and sulocitidil. The sub-MICs tested were as listed in Table 2. For raw data, see Figure S2.

Table 5. Broad-Spectrum Efficacy of Hits48

| compound (1 μL of mM) | B. cereus | M. luteus | B. subtilis | E. aerogenes | C. freundii | S. liquefaciens | V. natriegens | A. calcoaceticus | E. coli |
|-----------------------|------------|------------|-------------|--------------|-------------|----------------|--------------|----------------|--------|
| oxiconazole           | 0.41       | 0.063*     | 0.41*       |              |             |                |              | 0.83           | 3.30   |
| sulocitidil           | 0.41       | 0.21*      | 1.65        | 3.30         | 1.65        | 0.83           |              | 0.83           | 3.30   |
| hexestrol             | 0.83       | 0.21*      | 0.83*       |              | 0.41*       |                |              | 0.83           | 1.65   |
| dichlorophen          | 0.41       | 0.41*      | 1.65        |              | 0.83        | 0.83           |              | 0.83           | 1.65   |
| bithionol             | 0.013      | 0.025      | 0.013*      | 1.65         | 0.025       | 0.21           |              | 3.30           | 0.013  |

“All hits and 10 serial 2-fold dilutions of each hit were tested on various bacterial species grown on solid media. All MICs are reported in mM. One microliter of the stock, 10 serial dilutions, and DMSO were each spotted immediately after spreading 100 μL of each bacterial strain. Dashes indicate a lack of inhibitory efficacy. Asterisk indicates that MIC was equal to or at a lower concentration than on B. cereus, showing the hit’s increased efficacy on the inhibition of that bacterial species. Underlined are the names of Gram-negative bacterial species.
pharmacokinetics of this drug has been fully established in humans, hexestrol’s relatively low cytotoxicity in relation to the other hits suggests its promise as a drug used in combination with suloctidil at decreased concentrations to reduce its adverse effects and heighten its effects.30,32,33 Furthermore, hexestrol exhibited efficacy at equal or lower concentrations relative to its effects on B. cereus on four other bacterial strains tested, suggesting its broad-spectrum efficacy on various species of bacteria.

The present study analyzed the many facets of the hits in both solid and liquid contexts. However, further study is necessary on in vivo models such as flies or rats to move the application of these drugs toward human treatment. Additionally, the decreased efficacy of most of our hits on B. subtilis warrants concern for the applicability of the results to B. anthracis. While B. cereus is a commonly used surrogate to B. anthracis, the drugs must be tested directly on B. anthracis to confirm its efficacy on the pathogen responsible for the disease of concern. Finally, hits such as suloctidil showed tremendous efficacy in combinations well diluted beyond its sub-MIC. While we hypothesize that all of our hits target bacterial membrane, we also hypothesize that their combinations result in the synergized efficacy because they target different constituents of the bacterial membrane, such as phospholipids and proteins. Moreover, our drug combinations could also result in the synergized side effects in vivo. Further tests of combinations at lower concentrations must be conducted to test for the efficacy of the hits at lower levels of toxicity.

## CONCLUSIONS

The present study strived to identify potential repurposing opportunities to treat the seriously fatal anthrax disease by studying clinically approved drugs in the Johns Hopkins Clinical Compound Library. The five drugs of interest show prominent efficacy against B. cereus in various contexts and beg further investigation directly on B. anthracis and in vivo. In the face of a shortage of current treatments as well as a lack of a decisive treatment for the disease in all of its forms, there is great potential in drug repurposing as a more efficient method of discovering an improved cure for anthrax disease.

## METHODS

**Bacteria and Growth Conditions.** B. cereus (ATCC 14579) was grown in lysogeny broth (LB) at 37 °C. Subsequently, the liquid microbial culture was grown from a single colony by shaking at 200 rpm for a period of 16 h prior to the beginning of the experiment.

**Preliminary Screenings on Solid Media.** Three preliminary screenings were conducted on large Petri dishes (d = 150 mm). During the initial screening, a multichannel pipette was used to place 1 μL of each of the 3.3 mM drug stock in the 96-well JHCCl plates on solid media immediately after spreading them with B. cereus. Each dish screened one 96-well JHCCl plate. For all tests, slight impressions were made on the agar by the pipette tips to mark the locations of drug placement.

Hits were identified based on the criterion that the zone of inhibition produced by the drug was significantly larger and deeper than that of DMSO. All drugs that produced zones of inhibition of comparable size or depth to that of DMSO were eliminated. Depth of zones of inhibition was quantified by dividing the pixel count of each hit’s zone of inhibition by pixels in DMSO’s zone of inhibition after removing background noise.34 All images were captured by the iPhone 7 or 8 camera (12-MP wide-angle f/1.8 aperture)32,35 of the authors. ImageJ was used to quantify the size and depth of zones of inhibition on bacterial lawns.34

Two more preliminary screenings of 66 and 18 drugs, respectively, were conducted to confirm drugs that matched our criterion in the first screening. Drugs that did not repeatedly meet the criterion had already been reported as having antibacterial properties or were known to cause immunosuppression in patients who were not considered for the repurposing opportunities and eliminated, leaving us with five hits: oxiconazole, dichlorphen, suloctidil, bithionol, and hexestrol.

**Minimum Inhibitory Concentration (MIC) Tests.** Subsequent screens of our hits on solid media were conducted on individual Petri dishes for each drug (d = 85 mm). The minimal inhibitory concentration for each hit was tested by varying the concentration of the drug and the number of bacteria on which the drug was placed.

To study the efficacy of varying concentrations of each drug, 10 2-fold serial dilutions of each 3.3 mM drug stock were made. For each hit, 1 μL of the 3.3 mM stock, 10 dilutions, and DMSO were placed on one plate. MICs were defined as the lowest concentration of each drug at which the zone of inhibition was larger than that of DMSO.

MICs were also determined on incrementally increasing volumes of bacteria. Liquid media (108.3, 116.7, and 125 μL) containing B. cereus were spread onto individual plates for each hit, on which 1 μL of the 3.3 mM stock, 10 dilutions, and DMSO were placed. The same method of identifying MIC was used for all volumes of bacteria on which hits were placed.

Liquid assays of hits were performed at 37 °C for 12 h with readings every 10 min, with shaking prior to each reading. Each well contained 100 μL of LB media containing approximately 8.0 × 10⁸ cells (McFarland’s scale, OD at 600 nm = 0.1) with various drug concentrations. An MIC of a hit was defined as the lowest tested concentration that consistently inhibited bacterial growth in a significant manner. One microliter of DMSO was placed in 12 wells as a negative control, and uncontaminated LB media was placed in 12 wells and referenced as a negative control. The plates were grown with constant shaking at 37 °C, and the absorbance was measured by a SpectraMax Plus Microplate Reader (Molecular Devices) every 1000 s for a period of 12 h. The numerical OD₆₀₀ measurements included four decimal places.

**Assessing the Efficacy of Combined Sub-MICs of Hits.** Hits were combined at sub-MIC to test for additive or synergistic efficacy in both solid and liquid screenings. A hit’s sub-MIC on solid media was defined as the highest concentration at which the inhibitory effect was comparable to DMSO in area and intensity according to ImageJ analysis.34 For tests on solid media, stock solutions of hits were combined and diluted in DMSO to their individual sub-MICs, a concentration 2-fold dilution lower than their individual MICs. Three 1 μL volumes of each combined mixture were spotted alongside 1 μL of DMSO and 1 μL of each individual drug’s sub-MIC to confirm their lack of inhibition independently (Figure 4a).

Sub-MIC in the liquid was defined as the lowest concentration of the hit at which bacterial growth was not significantly different from the negative control, which was growth exhibited by untreated B. cereus. To test the efficacy of
combinations of hits in liquid assays, 1 μL of each drug’s sub-
MIC was combined in wells of the same conditions used in the Minimum Inhibitory Concentration (MIC) Tests section.

**Broad-Spectrum Properties of Hits.** All hits were tested on various bacterial strains to see broad-spectrum efficacy. Bacterial strains used were *E. aerogenes* (ATCC 51697, TSB media, 36 °C), *M. luteus* (ATCC 4698, LB, 25 °C), *B. subtilis* (ATCC 6633, LB, 37 °C), *C. freundii* (ATCC 8090, TSB, 30 °C), *S. liquefaciens* (ATCC 27592, TSB, 30 °C), *V. natriegens* (ATCC 14048, BHI, 35 °C), E. coli (C600, LB, 37 °C), and streptomycin-sensitive *Acinetobacter calcoaceticus* (ATCC 31926, BHI, 37 °C). For all strains tested, 1 μL of the 3.3 mM stock, six serial dilutions, and DMSO were placed on a lawn of each bacterial overnight. Further serial dilutions were spotted for hits that exhibited inhibition at the sixth serial dilution (0.053 mM).

**Statistical Analysis and Calculations.** GraphPad Prism 7.0d was used for statistical analyses, the area under the curve (AUC) = 0.05 was used for all statistical analyses. Two-tailed t-tests were used to determine the significance of inhibition in liquid assays. Microsoft Excel was used to create polynomial trends on bacterial growth curves (R² > 0.98).

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03207.

Lack of synergistic efficacy of combinations on solid media (Figure S1); synergistic efficacy of combinations in the liquid assay (Figure S2); and lack of synergistic efficacy of combinations in the liquid assay (Figure S3) (PDF)

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**Author Contributions**

A.L. and M.M.S. designed the research; M.A., S.G., A.J., A.H., C.P., and S.A. performed the research; all analyzed the data and wrote the paper. M.A., S.G., A.J., A.H., and C.P. contributed equally.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

A.L. acknowledges support from The Kenneth T. and Eileen L. Norris Foundation. M.M.S. acknowledges support from the City of Hope Comprehensive Cancer Center through the KL2 Mentored Career Development Award Program of the Inland California Translational Consortium (GR720001).

**ABBREVIATIONS USED**

*B. cereus, Bacillus cereus; B. anthracis, Bacillus anthracis; ATCC, American Tissue Culture Collection; JHClL, Johns Hopkins Clinical Compound Library; OD, optical density; US FDA, United States Food and Drug Administration; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; CLSI, Clinical Laboratory Standards Institute; LB, lysogeny broth; TSB, tryptic soy broth; BHI, brain heart infusion; Enterobacter aerogenes, *E. aerogenes*; Micrococcus luteus, *M. luteus*; Escherichia coli, *E. coli*; Bacillus subtilis, *B. subtilis*; Citrobacter freundii, *C. freundii*; Serratia liquefaciens, *S. liquefaciens*; Vibrio natriegens, *V. natriegens*; Acinetobacter calcoaceticus, *A. calcoaceticus*; OXI, Oxiconazole; SDL, Sulcotidil; HEX, Hexestrol; DCL, Dichlorophen; BTN, Bithionol; CMIC, critical micelle concentration

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