High-Throughput Screen for Inhibitors of *Klebsiella pneumoniae* Virulence Using a *Tetrahymena pyriformis* Co-Culture Surrogate Host Model

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**ABSTRACT:** The continuing emergence of antibacterial resistance reduces the effectiveness of antibiotics and drives an ongoing search for effective replacements. Screening compound libraries for antibacterial activity in standard growth media has been extensively explored and may be showing diminishing returns. Inhibition of bacterial targets that are selectively important under in vivo (infection) conditions and, therefore, would be missed by conventional in vitro screens might be an alternative. Surrogate host models of infection, however, are often not suitable for high-throughput screens. Here, we adapted a medium-throughput *Tetrahymena pyriformis* surrogate host model that was successfully used to identify inhibitors of a hyperviscous *Klebsiella pneumoniae* strain to a high-throughput format and screened circa 1.2 million compounds. The screen was robust and identified confirmed hits from different chemical classes with potent inhibition of *K. pneumoniae* growth in the presence of *T. pyriformis* that lacked any appreciable direct antibacterial activity. Several of these appeared to inhibit capsule/mucoidy, which are key virulence factors in hypervirulent *K. pneumoniae*. A weakly antibacterial inhibitor of LpxC (essential for the synthesis of the lipid A moiety of lipopolysaccharides) also appeared to be more active in the presence of *T. pyriformis*, which is consistent with the role of LPS in virulence as well as viability in *K. pneumoniae*.

**INTRODUCTION**

The incidence of multi-drug-resistant pathogens is increasing in clinics, and the continued success of current therapeutic agents for the treatment of infections is in jeopardy. However, efforts devoted to finding new antibacterial therapeutics appear to be scarce overall. This results in part from difficulties in identifying compounds that can penetrate into key Gram-negative pathogens, a high bar for safety with new chemical scaffolds, and difficulties with development pathways as well as an apparent lack of financial incentives in the antibiotics field. Since the golden age of antibiotic discovery that identified the currently used classes of potent antibiotics, efforts have been split between the design of next-generation analogs of these known antibiotic scaffolds and the identification of new scaffolds that inhibit novel protein targets that are essential for growth. The former approach has been more successful, as expected, since the body of knowledge surrounding these scaffolds is higher and efforts can be directed toward a single issue such as the emergence of resistance mechanisms. Examples include tigecycline and, more recently, the novel monobactam LYS228, which is designed to have improved stability to many serine β-lactamases, as well as a range of new β-lactamase inhibitors that address losses in clinical coverage by earlier compounds.

Finding compounds in standard corporate compound libraries that inhibit novel antibacterial targets in vitro is often straightforward, but these typically require the optimization of several parameters such as solubility and compound permeation into bacterial cells while meeting a high bar for safety in new compounds. Inhibitors are found by in vitro screens for inhibition of purified protein targets or by bacterial whole-cell screens for growth inhibition or for other phenotypic readouts indicative of target inhibition. In vitro enzyme screening can be very sensitive, but many inhibitors then lack sufficient cellular activity. Whole-cell screens can yield low hit rates in cases of intrinsically resistant pathogens such as *Pseudomonas*...
\textit{aeruginosa} or \textit{Klebsiella pneumoniae}, and therefore, cells that are defective in permeability and/or efflux or otherwise engineered for screening sensitivity are often used.\textsuperscript{16,17} These approaches then resemble the in vitro biochemical screening paradigm, yielding compounds that still need substantial optimization to gain wild-type cellular activity. Nonetheless, advances have been made toward cellular potency with novel inhibitors, for example, those targeted to Gram-negative lipopolysaccharide (LPS) biosynthesis and assembly. LPS consists of three moieties: lipid A, which forms the outer leaflet of the Gram-negative outer membrane, where attached are a sugar core region and variable polysaccharide O-antigen that extends outward from the cell. Compounds targeting the lipid A biosynthetic enzyme LpxC have been identified and optimized to have potent enzymatic and cellular activity.\textsuperscript{18} This is the case even for some notably resistant pathogens such as \textit{P. aeruginosa} and \textit{K. pneumoniae},\textsuperscript{19–21} although to date, none of the compounds have proceeded through clinical trials. Using a creative approach exploring the derivatives of \(\beta\)-hairpin molecules like protegrin, inhibitors of the LPS transporter LptD have been identified with very potent \textit{P. aeruginosa} antibacterial activity.\textsuperscript{22,23} These have a narrower spectrum, are confined to Gram-negative pathogens, and, in some cases, are pathogen-specific (e.g., \textit{P. aeruginosa}). A broader antibacterial spectrum is desirable, but the paucity of novel broader-spectrum agents may increase support for the development of narrower-spectrum agents.

The screening approaches described above have been mined for some time, and there may be diminishing returns from traditional corporate chemical libraries by similar screening efforts. Many corporate chemical libraries do not appear to be enriched for molecules within a property space thought to be optimal as starting points for antibacterial discovery.\textsuperscript{12} Enriching libraries for such starting points may improve this situation. In the meantime, screens using approaches differing from previous efforts might be useful to discover novel mechanisms and new inhibitors. One avenue may be targets that are not essential for growth in standard media but are necessary for survival during infection or that impact the severity of infection (in vivo essential, conditionally essential, or virulence targets)\textsuperscript{24–26} and could presumably be exploited therapeutically. An interesting demonstration of this principle derives from studies with LpxC inhibitors whose antibacterial spectrum does not include some strains of \textit{Acinetobacter baumannii} since lipid A is not essential for the laboratory growth of some strains.\textsuperscript{30–32} However, LPS is an endotoxin and a recognized virulence factor important for survival of Gram-negative pathogens in the host.\textsuperscript{33} Consistent with this, inhibitors of LpxC protected mice from \textit{A. baumannii} infection despite their lack of intrinsic antibacterial activity,\textsuperscript{34} illustrating the concept of potentially therapeutic molecules that lack in vitro antibacterial activity in standard MIC testing.

One approach to exploring in vivo essential targets is to screen under conditions that are more representative of the host scenario. For example, specific growth media designed to reveal and screen a range of essential targets differing from those surveyed using standard nutrient-rich laboratory media have been described.\textsuperscript{25,26,28,29,36,38} Several surrogate hosts have also been used in low to medium throughput for determining virulence, including zebrafish,\textsuperscript{37} \textit{Galleria mellonella} (wax moth),\textsuperscript{38,39} \textit{Caenorhabditis elegans} (worms),\textsuperscript{40} and the single-cell amoeba \textit{Dictyostelium discoideum}, which is an environmental predator of bacteria that shares traits of macrophages.\textsuperscript{41–43} There has been increasing interest in the adaptation of surrogate models of infection to screen for antivirulence compounds for a range of bacteria including mycobacteria, \textit{P. aeruginosa}, \textit{Salmonella enterica}, and \textit{Legionella}.\textsuperscript{44–46} A creative adaptation was the use of a motile ciliate, \textit{Tetrahymena pyriformis}, in a medium-throughput screen for inhibitors of virulence in \textit{Klebsiella pneumoniae} and for assessing the virulence of \textit{Aeromonas}.\textsuperscript{48} The former tested compounds for their ability to inhibit pathways in \textit{K. pneumoniae} that were not essential for growth in vitro but were necessary to resist predation and, when inhibited, therefore prevented the continued multiplication of the \textit{K. pneumoniae} component of the co-culture system.\textsuperscript{37} The motility of \textit{T. pyriformis} allowed a \textit{T. pyriformis}/\textit{K. pneumoniae} co-culture model to be established in a broth-based screening plate system, and a small screen yielded novel compounds that appeared to block capsule synthesis and possibly lowered production of LPS in a hypermucoid strain of \textit{K. pneumoniae}. Hypermucoidy is associated with virulence\textsuperscript{49} and compounds identified from the surrogate host screen had activity in a mouse model of infection using the hypermucoid strain.\textsuperscript{37} The use of a clinically relevant hypermucoid \textit{K. pneumoniae} isolate in the screen is noteworthy and contrasts the trend in many recent phenotypic antibacterial screens to employ permeability-compromised mutants to increase assay sensitivity. The use of such mutants is partly a response to the notion that historic phenotypic antibacterial screens have already yielded classes of inhibitors with potent activity against wild-type strains. Therefore, defective mutants increase susceptibility and increase the odds in identifying novel starting points. That is, however, specifically geared to identify inhibitors of less relevant defective mutants, and in general, the required conversion of these hits into compounds with useful potency against wild-type pathogens has proven difficult. Since compound libraries have not been screened yet against many nontraditional targets such as virulence, it remains a possibility that a subset of inhibitors of these targets that exhibit potency against wild-type pathogens still exist in libraries. Recognizing that the targets important in the co-culture assay (such as capsule) play a role in human infections, the predatory pressure applied by \textit{T. pyriformis}, used for sensitive detection of inhibitors, could reflect to some extent the immune system, which may apply similar pressure. However, many surrogate models of infection have had limited applicability due to relatively low throughput. Here, we successfully adapted the \textit{T. pyriformis} co-culture screen for a large-scale full-deck compound screen of the Novartis corporate library (circa 1.2 M compounds at the time of the screen). Several \textit{T. pyriformis}-dependent hits were confirmed with potent activity against the virulent wild-type \textit{K. pneumoniae} hypermucoid strain. Intriguingly, a novel LpxC inhibitor with weak antibacterial activity was more active in vitro against \textit{K. pneumoniae} in the presence of \textit{T. pyriformis}, which is consistent with the role of lipid A (LPS) in virulence, as well as viability, of \textit{K. pneumoniae}.

\section*{RESULTS}

\textbf{Manual Pilot Screens.} The assay utilized here is based on that described in ref 47. Briefly, the screen employs a motile single-celled ciliate (\textit{T. pyriformis}) in a co-culture system in liquid broth with the bacterium \textit{K. pneumoniae}. The growth of the \textit{K. pneumoniae} component of this co-culture was selectively monitored by optical density (OD\textsubscript{500}). \textit{T. pyriformis} is a predator of \textit{K. pneumoniae}, and a ratio of \textit{T. pyriformis} to \textit{K. pneumoniae} cells was established in the co-culture system where the overnight growth curve of the \textit{K. pneumoniae} culture was only slightly reduced, reflecting a low but detectable level of predation by \textit{T. pyriformis}. This was referred to as the Tp
threshold (described in detail in the Supporting Information). At the threshold, any compound inhibiting a pathway in K. pneumoniae that was important for resisting predation would then render the K. pneumoniae population highly susceptible to T. pyriformis, preventing the K. pneumoniae culture from getting established or effectively multiplying. Since inhibitors are therefore identified simply by the reduction of measurable K. pneumoniae culture growth overnight in the co-culture system (due to predation), hits score the same as would standard antibacterial compounds. Frankly antibacterial compounds also register as hits by virtue of their direct inhibition of K. pneumoniae growth in the co-culture system, but these are separated from hits that lack direct antibacterial activity by simply counterscreening for inhibition of K. pneumoniae in the absence of T. pyriformis. This approach has been shown to identify compounds that inhibit the ability of K. pneumoniae cultures to resist predation by T. pyriformis (e.g., compound D1, used as a positive control here, and D41; Figure 1).

Prior to adapting the assay for a full-library high-throughput screen, we conducted a series of manual pilot screens to ensure that the assay was performing as expected in this mode. These screens covered a total of 23,000 compounds, which included a test library of 8800 compounds designed to represent the overall makeup of the full library including known promiscuous inhibitors/cytotoxic compounds, as well as circa 12,000 purified natural products. Depending on the assay cutoff values used across these pilot screens, hit rates ranged from 0.16 to 0.78% (Supporting Information). This implied that there would be an acceptable range of 2000−9000 hits expected from a full 1.2 million library screen. One of the hits in these pilot screens (compound 25) was impure according to LC−MS conducted using freshly dissolved powder, but the mass of a major impurity corresponded to a structure related to the control D1 and the more active compound D41 (compound B1; Figure 1). Treating compound 25 with methanol and 1 M NaOH yielded an almost-complete conversion to the mass consistent with structure B1, and this product was active in the assay (IC50 of 0.74 μM (37,000 Tp) and 0.11 μM (50,000 Tp)). Therefore, albeit indirectly, our pilot screen had identified an analog of previously reported active compounds. Basic substructure searches around D1 yielded additional active analogs (examples are compounds 34 and 35; Figure 1 and Table S1), as well as an inactive analog that bears resemblance to the inactive analog D0 reported in ref 47 (Di; Figure 1 and Table S1), indicating potential SAR tractability. Also identified were three salicylics as active in this pilot (compounds 9, 10, and 16; Table S1). This was interesting since salicylic acids are reported to be inhibitors of both capsule production50,51 and virulence52 in mucoid K. pneumoniae. Table S1 contains examples of hits from these manual screens (under origin = Manual Screen) with structures classified together with the hitlist from the HTS (described below).

High-Throughput Screening Approach. Following the pilot screens, the Tp/Kp assay was adapted (see Methods) for use in screening the entire Novartis compound deck (>1.2 million wells, circa 2009). After assay adaptation to automation and optimization, the diverse set of 8800 compounds that comprised part of the manual pilot screen described above was retested in high-throughput mode to compare the day-to-day variability of the assay as well as compare the hits identified in the HTS mode to those identified in the manual screen. Of the 19 compounds identified earlier, >75% were identified in high-throughput mode, providing confidence to initiate the HTS. The full Novartis deck of compounds was screened at 10 μM with an average Z′ score of 0.82 and a standard deviation of 0.14.53 There were ~3000 compounds with ≥30% inhibition of Kp growth in the primary Tp/Kp co-culture screening assay corresponding to a 0.25% hit rate, which is similar to the focused screen of 15,000 compounds run at Athelas, which yielded a 0.16% hit rate.57 This list was minimally triaged, and the IC50 and counterscreen (Kp antibacterial-only activity) analysis was done. In parallel, analytical QC was done by LC/MS on the DMSO stock solutions for this dose−response experiment. About 26% of the samples did not meet the standard QC criteria (identity by Mass-Spec and UV purity ≥ 85%). This failure rate is in the range of other campaigns run at the time (median failure rate of 20% with standard deviation of 8.0% calculated on 47 campaigns). From the dose−response experiment, approximately 900 compounds were validated with activity only in the Tp/Kp primary assay. From this, 146 compounds with extrapolated or validated IC50 values of <10 μM and a differential activity between the Tp/Kp and Kp-only assays were selected for follow-up studies. Hit confirmation for these compounds was then done using solutions of freshly dissolved powder. Powder inventory was available for 140 of the selected compounds, and these were used for Tp/Kp and Kp-only IC50 determinations with 20 μM as the top concentration. A D1 control was included in each run, and the IC50 values for hits were normalized using that day’s value for D1 compared to an average D1 value derived from several earlier test runs in order to assist in rank-ordering across runs. The median IC50 for D1 across 28 hit confirmation runs was 5.7 with a standard deviation of 0.3. For the purposes of presenting data from the manual pilot screens, SAR expansions, and the HTS all in Table S1, we report the non-normalized values. Each compound available as powder was also tested for K. pneumoniae (Kp) antibacterial activity in

Figure 1. Structures of inhibitors identified in the K. pneumoniae/T. pyriformis co-culture assay. D1 and D41 in the figure above have been redrawn and previously reported.47 Di is an inactive analog of D1, which was identified when a set of D1 analogs was screened. Compound 25 was identified in a pilot screen but found to be unstable. Treating compound 25 with methanol and 1 N NaOH yielded an almost complete conversion to the mass consistent with structure B1, and this product was active in the assay (IC50 of 0.74 μM (37,000 Tp) and 0.11 μM (50,000 Tp)). Therefore, albeit indirectly, our pilot screen had identified an analog of previously reported active compounds. Basic substructure searches around D1 yielded additional active analogs (examples are compounds 34 and 35; Figure 1 and Table S1), as well as an inactive analog that bears resemblance to the inactive analog D0 reported in ref 47 (Di; Figure 1 and Table S1), indicating potential SAR tractability. Also identified were three salicylics as active in this pilot (compounds 9, 10, and 16; Table S1). This was interesting since salicylic acids are reported to be inhibitors of both capsule production50,51 and virulence52 in mucoid K. pneumoniae. Table S1 contains examples of hits from these manual screens (under origin = Manual Screen) with structures classified together with the hitlist from the HTS (described below).
Table 1. Data Summary for Representatives of Various Structural Classes Identified in a High-Throughput Screen Using a T. pyriformis/K. pneumoniae Co-culture Model

| compound | structure class | group | IC_{50} (μM) | MIC (μg/mL) | cytotoxicity IC_{50} (μM) | RBC hemolysis HC_{50} (μM) [max % hemolysis] |
|----------|----------------|-------|---------------|--------------|-------------------------|----------------------------------|
|          |                |       | Tp/Kp | Kp | Kp | Sa    |                                 |                                   |
| compound 187 | Cluster-1 1 | 11 | 0.16 >20 | >128 >128 | >558.95 | 128 [2.5] |
| compound 166 | Cluster-2 | 8 | 1.30 >20 | >128 >128 | >584.47 | 128 [2.5] |
| compound 185 | Cluster-3 | 3 | 0.85 >20 | >128 >128 | 16.47 | 128 [4.5] |
| compound 182 | Cluster-4 | 3 | 0.42 >20 | >128 >128 | 75.36 | 128 [2.4] |
| compound 62 | Cluster-A1 | 12 | 0.001 >20 | >32 >32 | >210.2 | 128 [11.7] |
| compound 63 | Cluster-A1 | 12 | 0.11 >20 | >128 >128 | 34.35 | 64 [8.3] |
| compound 64 | Cluster-A1 | 12 | 0.12 >20 | >128 >128 | >385.54 | 128 [14.9] |
| compound 65 | Cluster-A1 | 12 | 0.07 >20 | >128 >128 | >168.42 | 128 [1.5] |
| compound 67 | Cluster-A1 | 12 | 0.49 >20 | >128 >128 | >556.52 | 128 [1.5] |
| compound 68 | Cluster-A1 | 12 | 0.91 >20 | >128 >128 | >347.83 | 128 [2.2] |
| compound 70 | Cluster-A1 | 12 | 1.10 >20 | >128 >128 | >366.76 | 128 [17.6] |
| compound 74 | Cluster-A1 | 12 | 0.42 >20 | >128 >128 | >360.56 | 128 [26.9] |
| compound 170 | Cluster-A2 | 7 | 2.20 >13.73 | 64 8 | 102.15 | 128 [1.9] |
| compound 191 | Cluster-C1 | 4 | 3.22 >20 | >128 >0.06 | 35.84 | 128 [2.2] |
| compound 79 | Class-A | 5 | 1.33 >20 | >128 >128 | 341.42 | 128 [40.6] |
| compound 69 | Class-B | 6 | 0.05 >20 | >128 >128 | >291 | 128 [4.1] |
| compound 186 | Class-C | 3 | 5.40 >20 | >128 2 | 8.54 | ND |
| compound 73 | Singletons | 10 | 1.10 >20 | >128 >128 | >507.94 | 128 [2.8] |
| compound 82 | Singletons | 10 | 2.58 >20 | >128 >128 | >488.55 | 128 [7.4] |
| compound 180 | Singletons | 10 | 1.14 >20 | 4 >128 | 69.42 | ND |

*aChemical structures are shown in Figure 2. *bOf publishable compounds (public domain) from Table S1.

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single point at 128 μg/mL, and the MIC endpoint was then determined for any compounds that were active at this level. MIC values for a Gram-positive control Staphylococcus aureus were also determined. It should be noted that not all compounds were predicted to have high solubility, so MIC values >128 μg/mL should be considered tentative. To give preliminary information on potential for off-target effects, cytotoxicity in K562 cells and red blood cell hemolysis (an indicator of membrane disruption) were also determined for most compounds. A summary of data for all compounds with publishable structures and compounds. A structure grid containing all structures from this paper is included in Figure S1 and grouped based on structural classification. The classification recognizes small well-defined clusters as well as compounds that can be loosely grouped into two superclasses based on generic framework characteristics: (A) compounds containing extended aromatic systems and (B) compounds where two aromatic systems are separated by a flexible linker. A third superclass (C) contained natural product-like structures.

Consistent with our ability to identify analogs of the control compound D1 in pilot screens, six additional small heteroaryl analogs of D1 were present in the HTS hitlist (Cluster-1 in Table S1, example compound 187 in Table 1 and Figure 2). Another notable cluster consisted of thiazole-acetamide motifs (Cluster-A1, part of superclass A; compounds 62, 63, 64, 65, 67, 68, 70, and 74 in Table 1 and Figure 2), with some examples being potent in the surrogate host model system while lacking appreciable antibacterial activity against *K. pneumoniae*, cytotoxicity against K562 cells, or red blood cell hemolysis. Two of these active examples, compound 62 and compound 64, were resynthesized at Novartis (characterization details included in the Supporting Information). When IC_{50} values were determined side by side on the same assay plate with Tp set at 25000, the archived compound 62 powder yielded an IC_{50} of 0.0017 μM compared to 0.00067 μM for the resynthesis batch (D1 control at 2.316 μM), and the archived compound 64 powder yielded an IC_{50} of 0.3616 μM compared to 0.1855 μM for the resynthesis batch (D1 control at 2.734). This close agreement between the potency of archived powders and in-house resynthesis batches increased confidence in associating the thiazole-acetamide structures with activity in the Tp/Kp assay. A substructure search based on the thiazole-acetamide motif yielded several active analogs; examples with publishable structures that passed the analytical QC (as described above) are included in Table S1 (Cluster-A1 and origin SSS). An extremely potent hit from the HTS with a large differential between Tp/Kp activity and Kp-only activity was fosmidomycin (IC_{50} of <0.0781 μM for Tp/Kp and >20 μM for Kp only). Fosmidomycin is a natural product inhibitor of the DXP-reductoisomerase step of the non-mevalonate isoprenoid biosynthetic pathway. Commercially obtained fosmidomycin recapitulated the potent IC_{50} in the Tp/Kp assay (IC_{50} values of 0.0010, 0.0016, and 0.0043 μM for three independent assays, with the Kp only values all being >10 μM). Fosmidomycin did not initially appear to have strong antibacterial activity against *K. pneumoniae* NB29017 via broth-based assays. Growth inhibition appeared to occur after a period of initial growth, and the resulting turbidity interfered with MIC determinations (therefore, the MIC determined was called as >128 μg/mL since some turbidity was visible in all wells). However, potent antibacterial activity was observed with an agar MIC approach, which avoided this turbidity issue (MIC = 0.5 μg/mL). Qualitatively, when cells grown in liquid culture with fosmidomycin were supplied to the co-culture system with Tp, they appeared to be quickly cleared. This suggested that fosmidomycin exposure in broth yielded a population of dead/dying cells that were readily ingested by Tp. Therefore, the Tp/Kp assay may sensitively detect some slower-
acting antibacterial compounds and/or inhibitors of certain essential pathways.

**Effect of Compounds on Colony/Cell Morphology.** The control compound D1 used throughout these studies lacked antibacterial activity and was previously proposed to inhibit the synthesis of capsule (as measured by Western blot) and possibly lipopolysaccharide (LPS) in the mucoid *K. pneumoniae* strain NB29017. Therefore, we tested a representative selection of compounds and D1 in a qualitative assay for inhibition of the mucoid appearance of NB29017 colonies on agar plates. Briefly, compounds were incorporated into solid LB agar medium, and *K. pneumoniae* NB29017 cells were streaked for isolated colonies. NB29017 appears visibly mucoid on plates, and a reduction in this phenotype was visible by eye. The appearance of colonies after overnight growth in the presence of various levels of compound was qualitatively scored from 4 (mucoid) to 0 (nonmucoid appearance). These scores are shown in Table 2.

Many compounds, including D1, clearly reduced the mucoid appearance of colonies, suggesting that their activity in the Tp/Kp assay was at least in part based on interference with synthesis of capsule or other cell surface components. Cells of *K. pneumoniae* NB29017 grown in SM medium with or without compound 62 or the control compound D1 (both at 32 μg/mL) were examined by electron microscopy (Figure 3). Untreated cells exhibited the characteristic appearance of polysaccharide material at the surface while, as was consistent with previous reports, cells treated with D1 were largely devoid of this but still appeared to produce small amounts of surface material. Cells treated with compound 62 were devoid of any surface material, with a smooth appearance of the cell surface and possibly other effects on the cell envelope, again suggesting inhibition of a target related to cell surface polysaccharides, possibly including capsule.

**Tp/Kp Surrogate Model Using Cell Surface-Defective Mutants of K. pneumoniae.** Cell-surface polysaccharides, including capsule, are implicated in a range of mechanisms mediating survival and immune evasion in the host, including resistance to phagocytosis. Inhibition of capsule/hyper-
Compounds as Tested on Solid Agar Medium

viscosity appeared to be a primary mechanism being screened for in the Tp/Kp assay. Indeed, other surrogate models have shown capsule to be an important factor including a Galleria mellonella infection model and D. discoideum in studies using the same mucoid K. pneumoniae strain used here. Loss of capsule in this strain was also shown to increase phagocytosis by alveolar macrophages and affect virulence in a mouse infection model.

Along with capsule, the LPS of K. pneumoniae is also important for virulence. Disruption of certain genes involved in LPS biosynthesis such as wbbM, involved in O-antigen synthesis, and waaQ, involved in the biosynthesis of the LPS sugar core region, disrupted both LPS and capsule production. It was previously shown that K. pneumoniae NB29017 mutants having cell surface defects via mutation of the genes wbbM, waaQ, or rmpA, which regulates hyperviscosity, were not virulent in a mouse infection model and were cleared rapidly from Tp co-cultures established at a Tp density where the wild-type bacteria could resist predation. Here, we established a Tp/Kp screening assay with a cell surface-defective wbbM mutant, but the Tp threshold ranged from 4625–18,500 Tp/mL. This was lower than the typical 25,000–37,000 Tp/mL that was optimal for the wild-type K. pneumoniae, reflecting the reduced ability of the mutant to resist predation. This assay should not be able to detect a specific inhibitor of wbbM since this target is inactivated. Intriguingly, activity of the putative capsule inhibitor D1 was largely retained in the Tp/Kp wbbM assay setup (Table S2).

Furthermore, the structurally related compounds 34 and 35 (Cluster-1) also retained activity. The most potent compounds identified from the high-throughput screen, compound 62 and the related compound 64 (Cluster-A1), also retained potency in this assay format (Table S2). This suggests that although disruption of wbbM reduces capsule and attenuates virulence in this hypermucoid strain of K. pneumoniae, the target of these compounds is unlikely to be WbbM (at least in isolation) or possibly certain pathway enzymes directly downstream of WbbM.

**Differential Activity of an LpxC Inhibitor in the Tp/Kp Assay.** Unlike capsular polysaccharides or O-antigens, the lipid A moiety of LPS is essential for growth in K. pneumoniae in standard laboratory media. However, the well-characterized role of LPS in virulence and its potential relationship to capsule raised the interesting possibility that antibacterial inhibitors of lipid A biosynthesis might appear more active in the presence of Tp due to effects on the cell surface emanating from lipid A depletion, which would likely begin to occur at sub-MIC compound exposures. One hit included in Table S1 was an unpublished LpxC inhibitor from a historic LpxC program that had an IC_{50} of 40 nM against purified P. aeruginosa LpxC (compound 180). This compound made it through the screening funnel since it had a significant differential activity in the Tp/Kp vs Kp-only screening assays. However, it also showed cytotoxicity according to the K562 cell line assay and was not further studied from fresh powder. Therefore, a better-characterized LpxC inhibitor analog of compound 180, designated as compound 1, was tested in the Tp/Kp assay. Compound 1 was a more potent inhibitor of the LpxC enzyme (1 nM IC_{50} vs P. aeruginosa LpxC) and had potent antibacterial activity against P. aeruginosa but was only weakly antibacterial against K. pneumoniae strain NB29017 (standard MIC of 32 μg/mL). Two independent runs using compound 1 yielded Tp/Kp IC_{50} values of 4.78 and 2.15 μM and Kp-only values of >216 μM for fold differences of >45 and >100, respectively (Figures S2 and S3). Within these runs, the potent antibiotic ceftazidime (CAZ) yielded Tp/Kp IC_{50} values of

### Table 2. Qualitative Assessment of Inhibition of Mucoid Appearance of K. pneumoniae NB29017 Colonies by Several Compounds as Tested on Solid Agar Medium

| compound    | class      | Tp/Kp IC_{50} (μg/mL) | Kp MIC(μg/mL) | qualitative score^{b} on solid medium |
|-------------|------------|------------------------|---------------|---------------------------------------|
| D1          | control    | 0.54                   | ND            | 0 μg/mL 1 μg/mL 2 μg/mL 4 μg/mL 8 μg/mL 16 μg/mL |
| compound 62 | Cluster-A1 | 0.001                  | >32           | 4 3 slight 1                           |
| compound 63 | Cluster-A1 | 0.04                   | >128          | 4 1 0 0 0                             |
| compound 64 | Cluster-A1 | 0.04                   | >128          | 4 0 0 0 0                             |
| compound 65 | Cluster-A1 | 0.03                   | >128          | 4 2 2 2 2                             |
| compound 66 | Cluster-A1 | 0.11                   | >128          | 4 1 0 0 0                             |
| compound 67 | Cluster-A1 | 0.33                   | >128          | 4 3 3 1 1                             |
| compound 68 | Cluster-A1 | 0.38                   | >128          | 4 3 1 0 0                             |
| compound 69 | Class-A    | 0.47                   | >128          | 4 2 2 2 2                             |
| compound 70 | Class-B    | 0.02                   | >128          | 4 0 0 0 0                             |
| compound 73 | singletons | 0.28                   | >128          | 4 4 3 3 2                             |
| compound 74 | Class-A    | 0.13                   | >128          | 4 1 0 0 0                             |
| compound 75 | Class-A    | 0.02                   | >128          | 4 0 0 0 0                             |
| compound 76 | singletons | 0.68                   | >128          | 4 4 3 3 2                             |

Values expressed here as μg/mL for comparison to agar values. A score of 4 is fully mucoid, and a score of 0 is nonmucoid as assessed by eye.

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**Figure 3.** Electron micrographs of untreated mucoid K. pneumoniae strain NB29017 (A) or NB29017 grown in the presence of D1 (B) or compound 62 (C).
0.006 μM (both) and Kp-only IC₅₀ values of 0.15 and 0.043 μM, giving a much smaller average fold difference of only 4.5-fold. Therefore, there may be some effect on susceptibility to predation that occurs at exposure levels below their MIC for some antibacterial compounds (e.g., CAZ) but this appears to be very pronounced in the case of compound 1(−), possibly reflecting an impact on virulence in addition to frank antibacterial activity.

Figure 4. Tp/Kp screening campaign compared with three previously published high-throughput screens for activity against P. aeruginosa (efflux mutant) and for cytotoxicity against yeast and a human HaCat cell line.12 Hits for Tp/Kp were called at 30% inhibition; hits in the other three campaigns were called at 50% inhibition since screening concentrations were higher (20–40 uM vs 10 uM). (A) Primary hit rate data for sets of compounds binned by clogD. (B) Pie charts for the four growth-inhibition profiles in Tp/Kp and P. aeruginosa (efflux mutant) screening campaigns, colored by the hit status in yeast and HaCat cytotoxicity screens. All pies are sized by number of records except for bottom right. Top left, subset of compounds identified as primary hits in both Tp/Kp and Pa screens; top right, subset of compounds that only hit in Tp/Kp; bottom left, subset of compounds that only hit in Pa; bottom right, 0.5 M compounds that did not hit in either Tp/Kp or Pa.
**DISCUSSION**

Sparse financial incentives for antibacterial drug discovery may be negatively impacting new antibacterial research efforts, but the need for new agents continues and will only increase. Along with next-generation versions of current drugs, new approaches to identify antibacterial therapies may also be needed. One example may be antivirulence or “in vivo essential” antibacterial targets. Surrogate host models could be important in these efforts, although many such models may be suitable mainly for small- to medium-throughput screens. However, a recent antivirulence screen employing HEK293 mammalian cells was used to interrogate a 220,000-compound library for inhibitors of the intercellular spread of *Burkholderia*, supporting the potential of larger-scale screening efforts. Single-celled environmental predators of bacteria (e.g., *D. discoideum*) represent another approach, and the extension of this idea to *T. pyriformis*, which is motile in liquid culture, suggested the feasibility of very large library screens (>1,000,000 compounds). Here, we adapted the screen to this scale, and to our knowledge, this is the largest such screen reported to date. This assay is by extension also suited to multiple screens of smaller libraries designed to canvass a range of chemical property space or natural products.

Cell surface polysaccharides (e.g., capsule and LPS) are virulence factors in *K. pneumoniae*, and the Tp/Kp assay appears to have functioned well in identifying inhibitors affecting these pathways. Hits included salicylic acids, which are known capsule and virulence inhibitors in *K. pneumoniae*. Several analogs of inhibitors that appeared in previous lower-throughput screens (e.g., D1) were also identified along with additional chemical series and singletons. *Table S1* and associated *Figure S1* show the chemical space covered in this hit-finding campaign with classification of the structures and additional notes, including similarities of compounds to known drugs and indication of some compounds containing substrates that could covalently modify target proteins. The identification of apparently potent compounds such as compound 62 is of interest since the *K. pneumoniae* strain used here was not a hypersensitized lab strain but rather a clinically relevant hypermucoviscous isolate. Therefore, different approaches may yield new subsets of compounds where some have an ability to sufficiently penetrate into recalcitrant pathogens and exhibit cellular activity. Alternatively, inhibition of these targets under conditions of the screen, where the target pathway is very important for bacterial survival, may be sensitively detected. This condition-specific activity may translate to the scenario of the immune system at certain sites of infection, and such inhibitors would not be found in typical growth-inhibition screens.

A comparison of the primary hitlist from the Tp/Kp high-throughput screen with traditional antibacterial high-throughput screens builds confidence in the quality of the results. First, the primary hit rate for subsets of compounds binned by their clogD exhibits a similar profile for the Tp/Kp hitlist as observed for a previously published hitlist from a campaign targeting an efflux-defective *P. aeruginosa* strain (*Figure 4A*). The hit rate in the Gram(−) context is constant across the clogD range, whereas viability screens of yeast and mammalian cells show an increase in hit rate with increased clogD. The similarity in the clogD profiles for Tp/Kp and the *P. aeruginosa* campaigns (which has also been observed in unpublished Gram(−) screening campaigns) gives some confidence that the assay captures the inhibition of intracellular targets of Kp, which require passage through the dual membrane. The Tp/Kp hit rate overall was lower than the *P. aeruginosa* hit rate. This could be due to a number of factors, including the fact that the *P. aeruginosa* screen was done in an efflux-mutant strain, making it easier to inhibit. Another hypothesis for the lower hit rate is that the chemical space of our compound archive was less populated in the subspace that is relevant for the targets and mechanisms assessed in this predation assay. Further support for the latter hypothesis comes from the observation of a relatively low overlap between the Tp/Kp and *P. aeruginosa* hitlists: only 19% of the Tp/Kp primary hits were also primary hits in the *P. aeruginosa* primary screen. *Figure 4B* shows the overlap between the two Gram(−) hitlists as well as an indication of the proportion of those hits that are also primary hits in the yeast and mammalian cytotoxicity screens. It is interesting to note that the hits that overlap between Tp/Kp and *P. aeruginosa* (top left) have a cytotoxicity profile that is similar to the unique *P. aeruginosa* hits (bottom left), whereas the unique Tp/Kp hits (top right) show a reduced cytotoxicity profile, with only 17% showing a cytotoxicity flag compared to 50% in the *P. aeruginosa*-unique hits. This different profile may again be related to different mechanisms being assessed in the predation screen. In addition, it may also reflect the notion that some potential virulence inhibitors that are highly cytotoxic may preferentially inhibit Tp directly or otherwise impede their ability to ingest Kp, thereby reducing their ability to prey on Kp and, correspondingly, the chances they would appear as screening hits.

The ability to genetically inactivate certain potential targets in the in vitro context may also be informative. For example, deletion of *wbbM* in *K. pneumoniae* NB29017 significantly reduced capsule production and attenuated the strain in the model, but we showed here that compound activity could still be evaluated in Tp/Kp assays with this mutant by using a lower Tp threshold. Presumably, compounds that specifically inhibited WbbM or related downstream pathway enzymes would lose all or most of their activity in the mutant-based assay since the target is missing. In a practical sense, this could allow for directed screening funnels to bin those inhibitors. Somewhat surprisingly, since compound exposure and *wbbM* deletion both resulted in similar morphological effects on colonies and cells (by EM), the control compound D1 and several other compounds such as 62 were active in this *Kp*wbbM*/Tp* co-culture assay. This suggests that their target(s) may be related to capsule production but could also affect other pathways. Hyperviscosity and capsule production per se may be independent processes, and our understanding of these processes is evolving. Furthermore, compound D1 was also previously shown to reduce the amount of intact LPS associated with the wild-type *K. pneumoniae* strain used in this assay in addition to the reduction of capsule, again suggesting a target or targets related to more than one pathway. It is also possible, although perhaps less likely, that some compounds act to some extent by disbursing capsule or other loosely bound material similar to capsule depolymerases. Given the potential impact of D1 and other compounds on multiple cell surface components/PATHWAYS, efforts to identify specific cellular targets for these would be a worthwhile effort.

Intriguingly, in view of the abovementioned report that the non-antibacterial compound D1 reduced the amount of LPS associated with *K. pneumoniae* cells, the LpxC inhibitor compound 1 (−) tested here was more potent in the presence of Tp. This may reflect the role of LPS in bacterial virulence as well as viability. Increased susceptibility of *K. pneumoniae* to
prevention by *T. pyriformis* occurred at LpxC inhibitor levels well below those required to block bacterial growth outright. LpxC inhibitors appear to engender a meaningful depletion of lipid A (LPS) levels in *K. pneumoniae* at sub-MIC levels, since they potentiate the activity of other antibiotics via disruption of the outer membrane permeability barrier. Similar observations have been made with *A. baumannii*, and LpxC inhibitors can protect mice from infection by *A. baumannii* strains where LPS is not essential for in vitro growth (where the LpxC inhibitor does not have a standard MIC). Therefore, the Tp/Kp assay may point to antivirulence effects of LpxC inhibitors against *K. pneumoniae* at sub-MIC levels that could conceivably be important in the context of certain infections, in turn suggesting that such inhibitors would be more potent therapeutically than in vitro susceptibility assays might indicate.

Proponents of antivirulence approaches suggest that these may be less likely to select for resistance and that pathogen specificity would be less apt to disrupt the microbiome. These concepts are debated; however, the value of any narrower-spectrum agents will likely reflect the perceived importance of the target pathogen at various points in time. The campaign here was terminated around 2010 based on a potential narrow spectrum related to the focus on *K. pneumoniae* hypermucoidy and lack of association of antibiotic resistance with hypermucoid/hypervirulent isolates. However, strong warnings are now being raised about the global dissemination of hypervirulent *K. pneumoniae* infections in the clinic and, of importance, in community settings, intersecting with the emergence of multi-drug resistance in these hypervirulent strains. Concern over this has prompted research into therapeutics targeting hypervirulent strains, including a vaccine candidate. Inhibitors of specific virulence factors may cover a narrower spectrum of pathogens and may be specific for certain infection sites based on the relative importance of the factor. A recent study showed that both hypermucoid and capsule-deficient subpopulations of *K. pneumoniae* are distributed among clinical isolates and that these are associated with different infection scenarios. Hypermucoid strains are highly virulent, disseminate effectively, and are associated with blood stream infection and drug resistance, whereas capsule-deficient mutants may have an advantage to persist in the bladder and appear to be drug-tolerant. The inhibitor D1 (and by extension some of the compounds identified here) appears to inhibit multiple cell surface pathways including capsule and LPS, and therefore, more work is necessary to understand their targets and whether/where this could be applicable therapeutically. This opens the possibility that although capsule/mucoidy may be an important factor in hypermucoid *K. pneumoniae*, the spectrum of these inhibitors could extend beyond *K. pneumoniae* via other more conserved pathways. Supporting this concept, D1 was reported to have efficacy against *P. aeruginosa* in an animal model of infection.

**CONCLUSIONS**

Here, we present a large-scale HTS conducted using a surrogate host model of infection. This yielded confirmed inhibitors from various chemical classes that, in some cases, appear to inhibit the production of important cell-surface virulence factors such as hyperviscosity and/or capsule. The assay was robust and reproducible in the HTS mode, and properties of the confirmed inhibitors (logD profile, lack of intrinsic antibacterial activity, and lack of cytotoxicity) are encouraging for follow-up work to attempt the identification of tractable targets. As conventional drugs continue to become ineffective against emerging pathogens such as MDR hypervirulent *K. pneumoniae* strains, approaches such as that described here as well as the compounds identified may warrant renewed attention.

**METHODS**

The hypervirulent, hyperviscous *K. pneumoniae* strain KP52142 (here designated as NB29017), its *wbbM* capsule defective mutant (NB29018), and *T. pyriformis* (CCAP1630/21, culture collection, SAMS Research Services, Argyll, PA37 1QA, UK) were previously described. *K. pneumoniae* NB29001 is ATCC 700603, a control strain for susceptibility testing. *P. aeruginosa* PAO1 ΔmexAB-oprM strain K1119 was previously described. Compounds D1 and D41 (compounds AT1H8534 and AT125956) were obtained from Athelas SA (Switzerland).

*T. pyriformis*/*K. pneumoniae* (Tp/Kp) Virulence Assay. A chemostat system for growth of *T. pyriformis* is described in detail in the Supporting Information and Figure S4. The assay used here is based on that previously described with modifications. The assay measures the ability of a *K. pneumoniae* NB29017 (Kp) culture to resist predation by *T. pyriformis* (Tp) when co-cultured, requiring the level of Tp cells included in the co-culture system to be optimized. This level (Tp threshold) is established by testing dilutions of Tp cells against a fixed level of Kp cells to establish when KP can uniformly resist predation (Supporting Information and Figure S5). The Tp cells grown in the chemostat were routinely monitored for fitness, and a typical Tp threshold used for compound screening assays was between 25,000 and 50,000 Tp per mL with the Kp cells fixed at a density of circa 5 × 10^8 cfu/mL. For daily manual pilot screens of smaller compound libraries or for determination of compound IC_{50} values (inhibition of Kp growth in the co-culture), a panel consisting of five types of plates were used. The first was a reduced threshold matrix plate that was set up as for the full threshold assay (Supporting Information and Figure S5) but covering only the range of Tp from 50,000 to 12,500 Tp/mL. This served as a control to ensure that the anticipated Tp/Kp levels based on the full threshold determination remained valid during compound screening/testing assays and that control compound activity was being sensitively detected. The second plate setup was the Tp/Kp assay plate set at the Tp threshold calculated the day before the assay. These were used either to screen compounds or to determine IC_{50} values. The third plate setup contained only Kp as a control to identify compounds with intrinsic antibacterial activity, which allowed for the binning of compounds into Tp-dependent (only active in the Tp/Kp plates) and non-Tp-dependent activity (active in Tp/Kp and Kp alone). For manual pilot screens, compounds provided in 384-well plates as 2 mM DMSO stocks were added twice to the Tp/Kp and Kp-only plates using a handheld 384-well pin replicator (2 × 0.1 μL into a total volume of 50 μL per well) to give a final concentration of 8 μM and 0.4% DMSO. For IC_{50} determinations, compound stocks were freshly prepared from powder in DMSO at 5 mM, and twofold dilution series were then prepared in DMSO and transferred to 384-well plates. Compounds were transferred twice by a pin replicator, which resulted in a starting concentration of 20 μM. If necessary, lower concentrations were prepared to achieve an endpoint. In some instances, IC_{50} determinations were carried out at two or three different Tp densities (including one above and one below the Tp threshold) to ensure adequate sensitivity and signal-to-noise ratio. The control compounds D1 and D41 were also included in IC_{50} plates (rows GHIK) as per the threshold assay plates. Plates
were then incubated overnight at 32 °C, and the OD_{600} was read using a SpectraMax plate reader. The IC_{50} curves were plotted, and IC_{50} calculations were done using GraphPad Prism.

**Tp/Kp High-Throughput Screen (HTS).** The Tp cultures were grown in a chemostat and monitored for fitness weekly as described above. The protocol for the large-scale screening of 1,225,157 wells using the Tp/Kp assay was a modified version of the small-scale assay described above. For the high-throughput effort, clear bottom plates were used (catalog no. 4336, Thermo Scientific Matrix) due to the unavailability of black screening plates in quantities sufficient for HTS. For the primary screen, only the Tp/Kp assay was run, with the Kp-only counterscreen being run during confirmation of the primary hits. Order-of-addition studies indicated that Kp could be added last, so this order was used to accommodate the use of a BSL2 biological safety cabinet during the large-scale effort. Each set of compounds was run using a 3 day screening protocol as follows: On day 1, 10 mL of Tp culture from the chemostat was diluted into 40 mL of Tp media and incubated overnight at 30 °C in tissue culture flasks. A single agar plate colony of Kp was inoculated into each of the two flasks containing 50 mL of SM media and incubated with shaking overnight at 37 °C. On day 2, the assay plates were prepared: compounds were diluted to a ratio of 1:20 in a compound plate using 1 μL of 2 mM compound in 90% DMSO stock to 19 μL of SM media (FlexDrop). This resulted in 100 μM source plates at 10X the final assay concentration. Note that the upper limit of DMSO tolerance was found to be approximately 0.5%, which limited the upper-compound screening level to 10 μM. Five microliters of SM medium was added to column 24 of the assay plates (FlexDrop). Five microliters of the diluted compounds were transferred to assay plates (PlateMate Plus; CyBi-Well). The overnight Kp culture was diluted to a ratio of 1:5, and the OD_{600} was read using a SpectraMax plate reader. The culture was then adjusted to 2.0 × 10^{10} (10X final concentration) in SM media, assuming that an OD_{600} of 1.0 was 1 × 10^{8} cfu/mL. The Tp cultures were pelleted at 1200g, and the pellets were suspended in SM medium. The cells were counted using a hemocytometer, and then the suspension was adjusted to ~46,875 cells/mL (1.25X) in SM medium. Forty microliters of Tp suspension was added to assay plate columns 1–24 (MultiDrop 1). Then, 5 μL of the 10X Kp suspension was added to columns 1–23 (MultiDrop 2, in the biological safety cabinet). The plates were then incubated overnight at 32 °C in cell culture plate racks, and on day 3, the plates were read (OD_{600}) using an Envision plate reader. Note that in order to increase the daily throughput, the final protocol was split into two separate but parallel pathways (A and B), each having its own compound transfer pipettor, the Tp-dispensing MultiDrop and the Envision plate reader (PerkinElmer). The two paths shared the compound dilution (FlexDrop) and the Kp-dispensing (MultiDrop) steps. In the data analysis phase, each path was analyzed separately, allowing pathway-specific data corrections when necessary. Also, the active control wells (column 24) simulated 100% inhibition by not adding any bacteria, resulting in the theoretical maximum possible light transmission through the well in the presence of only Tp, though it is worth noting that this transmission was greater than that seen in wells containing fully inhibitory concentrations of control compound. Note that under these conditions, the entire NVS corporate library of 1.2 million wells was screened in less than 4 weeks. Compound hits were validated by IC_{50} determination using an 8-point 2-fold dilution (10–0.078 μM), and the Kp-only counterscreen for direct antibacterial activity was conducted at this time using the same compound dilutions. As mentioned above, the DMSO tolerance of the Tp/Kp assay limited the upper concentration to 10 μM. Because the highest validation concentration was the same as the primary screening concentration, it was not possible to calculate the validation rate using the preferred criteria (i.e., compounds that had an IC_{50} of less than twice the primary screening concentration). Therefore, three methods of calculating the validation rate were used. The first method was to count all curves that were not constant, i.e., those that showed at least some activity at 10 μM or less. These curves essentially confirmed the primary result. This confirmation rate was 49.9%. The second method was to count all curves for which the extrapolated IC_{50} was less than twice the primary screening concentration. This approximated the preferred method. Extrapolated values were taken directly from Helios. To maximize the likelihood of an accurate extrapolation, the bottom plateau of the incomplete curve was fixed at 100% inhibition. This rate was 35.1%. The third method was to count all curves that showed an IC_{50} of less than 10 μM, though weaker compounds (i.e., <50% inhibition from the primary HTS) would not be captured here. These curves were from the more potent compounds. This rate was 17.5%.

**Effect of Compounds on Colony and Cell Morphology (Appearance of Mucoidy of K. pneumoniae NB29017)**. Componds were resuspended in DMSO to a concentration of 12.8 mg/mL. Compound was added to 5 mL of melted LB agar at concentrations ranging from 0–16 ug/mL, and each dilution was then added to six-well polystyrene culture plates. The agar was then allowed to solidify, and the mucoid K. pneumoniae wild-type strain NB29017 was streaked for direct antibacterial testing containing 50 mL of SM medium (see Supporting Information) with and without compounds D1 or NB29017. The MIC was determined using an 8-point 2-fold dilution (10^{-8}−10^{-5}) M). Each set of compounds (i.e., <50% inhibition from the primary HTS) would not be captured here. These curves were from the more potent compounds. This rate was 17.5%.

Cells were prepared for electron microscopy as follows: Strain NB29017 was grown overnight in 10 mL of SM medium (see Supporting Information) with and without compounds D1 or compound 62 at 16 ug/mL, and each dilution was then added to six-well polystyrene culture plates. The agar was then allowed to solidify, and the mucoid K. pneumoniae wild-type strain NB29017 was streaked for direct antibacterial testing containing 50 mL of SM medium (see Supporting Information) with and without compounds D1 or NB29017. The MIC was determined using an 8-point 2-fold dilution (10^{-8}−10^{-5}) M). Each set of compounds (i.e., <50% inhibition from the primary HTS) would not be captured here. These curves were from the more potent compounds. This rate was 17.5%.

**Antibacterial Susceptibility Testing and Determination of Cytotoxicity.** Antibacterial MICs and cytotoxicity in mammalian cell lines were done as previously described. Red blood cell hemolysis was determined using a 96-well plate format adapted from ref 87 as follows: erythrocytes were harvested from defibrinated sheep blood (Remel) by centrifugation at 1000g (approximately 4000 rpm) for 10 min. The cell pellet was resuspended in 1 mL of assay buffer (30 mM Tris–HCl (pH 7.5) and 150 mM KCl) and washed two more times in assay buffer. The supernatant was removed, and its volume was measured. The volume of the remaining pellet in the tube was then calculated by subtracting the supernatant volume.
from the total volume after resuspension. The suspended cells were then adjusted to a total volume equal to 10 times the pellet volume (the stock cell suspension). The working suspension was prepared by further diluting the stock suspension to 1:10 in assay buffer. Three-fold compound dilutions were prepared in polypropylene 96-well plates (NUNC). Ten test compounds were run (columns 1–10), with column 11 reserved for a negative control (such as ciprofloxacin) and column 12 reserved for the positive lysis control (Triton X-100). After filling all wells in rows B though H with 30 μL of DMSO, 45 μL of a 12.8 mg/mL stock was used to give a 128 μg/mL starting concentration in the assay (unless otherwise noted). The Triton X-100 stock was (100 mg/mL), and the ciprofloxacin stock was 6.4 mg/mL. Compounds were diluted 3-fold from rows A to H by transferring 15 μL into 30 μL. Two microliters of stock from the compound stock dilution plate was added into two 96-well V-bottom polypropylene plates using a multichannel pipettor. Two hundred milliliters of the red blood cell suspension was added to one of the 96-well polypropylene V-bottom plates (Hemolysis Assay Plate), and 200 μL of assay buffer was added to the second plate (Compound Background Plate). After incubating both plates at 37 °C for 1 h, they were centrifuged at 3000 rpm for 10 min, and 150 μL of the supernatant was transferred to a 96-well clear flat-bottom polystyrene plate. Released hemoglobin was measured with an absorbance of 540 nm with a SpectraMax plate reader. Data was processed using Molecular Devices Softmax software and Excel Fit 4 (IDBS). The % hemolysis was calculated as 100 × (abs (RBC + compound) − abs (compound alone))/average positive − average negative). A four-parameter logistic model or sigmoidal dose–response model was used for curve fitting to determine the HC₅₀ (concentration causing 50% hemolysis).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06633.

Results summary for manual screens using a T. pyriformis/ K. pneumoniae co-culture; characterization of resynthesized compounds 62 and 64; (Figure S1) chemical structures of compounds listed in Table S1; (Figure S2) differential activity of the LpxC inhibitor compound 1(−) between the Tp/Kp and Kp-only assays: assay plates; (Figure S3) differential activity of the LpxC inhibitor compound 1(−) between the Tp/Kp and Kp-only assays: IC₅₀ curves; installation and maintenance of a chemostat for growth of T. pyriformis; (Figure S4) chemostat setup for continuous growth of T. pyriformis; establishing the Tp threshold; (Figure S5) plate layout for determining a T. pyriformis threshold (PDF)

(Table S1) Compound hits from the Tp/Kp co-culture screen; (Table S2) impact of genetic deletion of wbbM on activity of selected compounds (XLSX)

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