High-Throughput Identification of Chemical Inhibitors of E. coli Group 2 Capsule Biogenesis as Anti-Virulence Agents

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Abstract

Rising antibiotic resistance among Escherichia coli, the leading cause of urinary tract infections (UTIs), has placed a new focus on molecular pathogenesis studies, aiming to identify new therapeutic targets. Anti-virulence agents are attractive as chemotherapeutics to attenuate an organism during disease but not necessarily during benign commensalism, thus decreasing the stress on beneficial microbial communities and lessening the emergence of resistance. We and others have demonstrated that the K antigen capsule of E. coli is a preeminent virulence determinant during UTI and more invasive diseases. Components of assembly and export are highly conserved among the major K antigen capsular types associated with UTI-causing E. coli and are distinct from the capsule biogenesis machinery of many commensal E. coli, making these attractive therapeutic targets. We conducted a screen for anti-capsular small molecules and identified an agent designated “C7” that blocks the production of K1 and K5 capsules, unrelated polysaccharide types among the Group 2–3 capsules. Herein lies proof-of-concept that this screen may be implemented with larger chemical libraries to identify second-generation small-molecule inhibitors of capsule biogenesis. These inhibitors will lead to a better understanding of capsule biogenesis and may represent a new class of therapeutics.

Introduction

Escherichia coli is the leading cause of community-acquired urinary tract infections (UTIs), producing over 80% of community-acquired UTI and at least 50% of nosocomial UTIs [1]. Twenty-five to forty percent of first-time community-acquired UTIs are followed by recurrences caused by the same clone of UPEC. In addition, E. coli also accounts for a significant proportion of sepsis and meningitis of the young and old, with the infections originating from the urinary tract or direct translocation from the gut into the bloodstream. With over 100 million UTIs occurring annually throughout the world, including more than 10 million cases in U.S. adolescents and adults (per NIDDK data, [2]), UPEC accounts for substantial medical costs and morbidity worldwide.

Accompanying the large use of antibiotics for UTI and other common infections like those of the respiratory tract has been rising antibiotic resistance among E. coli, resulting in many cases in multidrug resistant strains [3,4,5,6,7], and invigorating efforts to elucidate vulnerable targets in the molecular pathogenesis of infection. Of the oral therapies for community-acquired UTI and at least 50% of nosocomial UTIs [1].}

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Funding: PCS received support from National Institutes of Health (NIH) grant K08DK074443 and NIH Office of Research on Women's Health SCOR P50DK064540. CCG received support from a National Science Foundation (NSF) Facilitating Academic Careers in Engineering and Science (FACES) fellowship award and a Duke Children's Miracle Network grant and currently is a postdoctoral fellow of the Hartwell Foundation (http://www.thehartwellfoundation.org/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

E. coli Group 2 Capsule Biogenesis as Anti-Virulence Agents. PLoS ONE 5(7): e11642. doi:10.1371/journal.pone.0011642

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Citation: Goller CC, Seed PC (2010) High-Throughput Identification of Chemical Inhibitors of E. coli Group 2 Capsule Biogenesis as Anti-Virulence Agents. PLoS ONE 5(7): e11642. doi:10.1371/journal.pone.0011642

Editor: David M. Ojcius, University of California Merced, United States of America

Received May 14, 2010; Accepted June 22, 2010; Published July 19, 2010
Antimicrobial peptides (AP), including the cationic 3–5 kDa peptides called defensins, are abundant in the urine [17]. AP form pores in phospholipid bilayers but require access to the bacterial outer membrane for function [18]. The effectiveness of the innate immune response against bacteria such as *E. coli* may, however, be hindered by bacterial factors such as polysaccharide capsules. *E. coli* is also a well-recognized cause of urosepsis, and bacteria translocating from the urinary tract into the bloodstream are subject to most of these same assaults as enacted by the innate immune system of the urinary tract.

Capsules are well-established virulence factors for a variety of pathogens and serve to protect the cell from opsonophagocytosis and complement-mediated killing (reviewed in [19,20]). K capsules, also called K antigens, are enveloping structures composed of acidic, high-molecular-weight polysaccharides. Among UPEC, the K antigens K1, K5, K30, and K92 are most prevalent [21]. In recent work, Llobet et al. demonstrated that highly acidic polysaccharide capsules of *K. pneumoniae*, *P. aeruginosa*, and *S. pneumoniae* interact strongly with APs, acting as “sponges” to sequester and neutralize the APs [22]. Furthermore, we have found that K capsule contributes to multiple aspects of UTI pathogenesis, including intracellular replication [23], making inhibition of capsule biosynthesis a novel target for attenuation of UPEC virulence.

The Group 2 K capsule genes can be divided into three genetic regions: ASSEMBLY (I), SYNTHESIS (II), and EXPORT (III). Group 2 & 3 capsules require homologous ASSEMBLY and EXPORT proteins, but the genes for Group 3 are re-distributed among the Region I–III gene clusters. In addition, many of the components encoded in Regions I and III have homologues in other medically important bacteria such as *Neisseria meningitidis*, and these counterparts also participate in capsule biosynthesis. Among the *E. coli* Group 2 capsules, the K1 and K5 serotypes account for the majority of UPEC K clinical isolates. K1 is composed of 2,2,8,9 linked poly-Neu5Ac, and K5 contains repeating N-acetylgalactosamine and glucuronic acid units [24,25].

Genetic disruption of Group 2 and Group 3 capsule production results in predictable phenotypes, and determining if and where polysaccharide accumulates in the cell can point to where capsule biosynthesis is blocked by genetic mutation or chemical inhibition. Interruption of many points in the SYNTHESIS genes abrogates the accumulation of intra- and extracellular polymers and renders the organism insensitive to K1F phage, which requires surface capsule for entry [26,27].

Inhibiting K capsule production may sensitize the organism to a conventional antibiotic or component of the immune system. Proof-of-concept evidence comes from the demonstration that injection of purified K1 endosialidase prevented sepsis and meningitis after intraperitoneal infection of neonatal rats with *E. coli* K1 [28]. Endosialidase had no direct *in vitro* effect on *E. coli* K1 viability, but presumably removed K1 capsule *in vivo*, rendering the organism more susceptible to host immune factors and external stress, attenuating the infection. However, endosialidases have limited therapeutic applications due to their antigenicity, poor bioavailability, and potential action on sialidased host proteins and lipids with shared linkages as the capsular sialic acids [such as those present in neural tissues, reviewed in [29]]. Furthermore, endosialidase has a very narrow biochemical target range, limiting its application to specific K antigen types. Chemical inhibition of K capsule production may achieve similar therapeutic results without most of these limitations.

Exploiting the properties of K capsule-specific phage, we designed an innovative yet simple screen to uncover small molecule inhibitors of capsule biosynthesis. In this report, we highlight the potential of our approach by describing the identification and basic characterization of a novel agent designated “C7”. This agent is active (IC50 between 12.5–25 μM), blocks the production of K1 and K5 capsule biosynthesis and lacks obvious toxicity to cultured bladder epithelial cells. Beyond the scope of C7 and its analogues as potential capsule inhibitors, the knowledge gained through these studies will expedite future large screens for additional broad spectrum capsule inhibitors, elucidation of their molecular targets, and evaluation as anti-therapeutics.

**Results**

High-throughput screen for small compound inhibitors of capsule

Based on the observation that chemical inhibitors of multiple K type capsules could be identified, a high-throughput screen for inhibitors of capsule biosynthesis was developed in which the prototypic uropathogenic *Escherichia coli* strain UTI89 was grown in LB broth in 96-well plates in the presence of 100 μM compound or 1% DMSO vehicle followed by treatment with the K1 capsule specific phage K1F (K1F φ). Figure 1B depicts the screening process. A wild-type UTI89 K1 encapsulated strain grown in the presence of vehicle and treated with K1F phage at an OD600~0.1–0.2 was quickly lysed and served as a positive control. In contrast, a genetic capsule assembly mutant (UTI89 ΔkpsM) was resistant to K1F phage lysis and was used as a negative control. Compounds that affected capsule synthesis or assembly were predicted to produce bacterial resistance to phage. A large signal-to-noise ratio and low frequency of false positives make this a very robust assay. The Z’ factor, a measure of the suitability of a particular assay for use in a full-scale high-throughput screen, was determined to be 0.93 when performed in a 96 well microtiter plate format.

We expected one of multiple outcomes from the assay. First, chemical inhibitors of bacterial growth were expected to be present in most chemical libraries. An initial absorbance reading after ~1 hr of growth was used to determine those compounds that severely affected bacterial growth (arbitrarily defined as those with an OD600 less or equal to 0.05 after first time point and no increase thereafter). Compounds producing growth inhibition were not further analyzed. Of the compounds that did not inhibit bacterial growth but prevented phage lysis, we anticipated they would be categorized into two primary groups: 1) those affecting capsule biosynthesis (true positive) and 2) those inhibiting the phage lifecycle (false positive). The latter groups could be identified and eliminated by the secondary screen described later.

In total, 2,195 compounds from the Developmental Therapeutics Program at the National Cancer Institute were screened. Seventy five (3.41%) of the compounds produced significant inhibition of growth and were eliminated. Thirty five (1.59%) compounds inhibited K1F phage lysis in the 96 well plate format. However, only 9 of these 35 reproducibly inhibited phage lysis in a larger shaken tube format and these 9 were advanced into the secondary screening process. Table 1 summarizes the results of the initial high-throughput screen.

**Secondary assays**

Compounds that inhibited K1F phage lysis in the primary assay were further tested in secondary screens to distinguish between inhibition of capsule biosynthesis and inhibition of phage replication. The secondary screens capitalize on the knowledge that T7...
and K1F phage are genetically and physiologically closely related [27]. However, K1F phage entry requires K1 capsule, while T7 phage entry is inhibited by the capsule. As a result, only unencapsulated bacteria are subject to T7 infection and lysis [30].

The engineered K12:K1 hybrid strain called EV36 producing K1 capsule was grown in the presence of the putative capsule inhibitors and was then infected with T7 phage (T7φ). Compounds that sensitized the K12:K1 strain to T7 phage were deemed to affect capsule, and 2 of 9 compounds rendered EV36 sensitive to T7. The confirmation that these 2 compounds were effective in eliminating capsule in this well-characterized K1 hybrid strain is that it confirmed the capsule inhibition effects in an independent unrelated genetic background (Table 1).

Table 1. Summary of high-throughput screen.

|                          | N | % |
|--------------------------|---|---|
| Total compounds screened | 2195 | - |
| Compounds causing growth inhibition | 75/2195 | 3.42 |
| Inhibited K1 lysis       | 35/2195 | 1.59 |
| Sensitized K-12:K1 to T7 phage | 2/9 | 0.09 |
| Inhibited T7 lysis of K-12 strain | 7/9 | 0.32 |

doi:10.1371/journal.pone.0011642.t001
To further confirm that the effect of each compound was not due to inhibition of phage replication, the prototypic K-12 strain MG1653, which lacks K1 capsule and is readily susceptible to T7 phage infection, was grown in the respective compounds. Compounds inhibiting T7-mediated lysis of MG1653 were excluded as being phage-specific and not affecting capsule biogenesis. Several compounds inhibited T7 lysis of MG1653, indicating that they likely affected phage physiology (Table 1). Table 2 lists the agents that passed the primary screen and indicates the performance of each chemical in the secondary assays.

C7 as lead compound

In our initial screen, we identified 2 inhibitors of capsule biogenesis (Table 2). Although not previously described as an inhibitor of capsule biogenesis, NSC5550, also known as malachite green oxalate, produces metabolites with known toxicities to mammalian systems and was therefore not pursued further at this time. The other inhibitor, 2-(4-phenylphenyl)-benzo[g]quinoline-4-carboxylic acid (NSC136469), was investigated further. We designated this molecule as “C7” and pursued it as a prototype for a chemical inhibitor of capsule biogenesis. C7 reproducibly inhibited K1F phage lysis of UPEC K1 strain UTI89 in tests following the high throughput screen (Figure 2A), and the inhibition was found to be dose-dependent with the effect reaching saturation at ~25 μM C7 (p = 0.3731, 25 μM and 100 μM C7), producing ~50% inhibition of K1F phage lysis of UPEC at 12.5–25 μM (Figure 2A). C7 activity was also tested on the K12:K1 strain EV36, a reciprocal experiment of the K1F lysis inhibition. EV36 grown in the presence of 100 μM C7 was highly susceptible to T7 phage, suggesting that C7 inhibited K1 capsule production and allowed T7 to attach, enter, and lyse the target bacteria (Figure 2B). Next, we determined if C7 could inhibit a distinct non-K1 Group 2 K capsule-expressing strain. The UPEC K5 pylonephritis isolate DS17 was grown in the presence and absence of C7. Without C7, DS17 was readily lysed by K5 bacteriophage. In contrast, treatment with 100 μM C7 nearly completely inhibited K5 bacteriophage lysis (Figure 2C). In summary, the phage data suggests that C7 acts also on K5 capsule production/assembly and that C7 targets a convergent point in the production of these two different Group 2 polysaccharides. These data provide proof-of-principle that this simple and efficient high-throughput screen of a relatively limited compound library is able to yield candidate small molecule inhibitors of Group 2 capsule biogenesis.

Effect of C7 on capsule

Based on the results of our phage assays, we hypothesized that C7 acts on a step in capsule assembly or export that is common to both K1 and K5 capsule types. In order to localize the point of inhibition and the molecular target of C7, we sought to determine the phenotypic consequences of C7 treatment on capsule biogenesis, and whether C7 treatment resulted in capsule release, intracellular accumulation of polymer, or inhibition of synthesis. Whole cells and sonicates were used in agglutination and radial immunodiffusion assays using the H46 anti-K1 capsule polyclonal antiserum. Whereas wild-type and genetic capsule synthesis mutants were positive and negative for agglutination, respectively, C7 treated UTI89 did not agglutinate, and whole cell sonicates had only weak reactivity (Table 3). We next sought biochemical evidence that C7 inhibited capsule production in UPEC K1. Extracted polysaccharides obtained from whole cell sonicates or surface capsule released (by mild acid treatment (adapted from [31]), were treated with Bial’s orcinol reagent that reacts with pentoses [32]. Polysaccharides extracted from whole cell sonicates of C7 treated cells yielded significantly lower orcinol reactivity than extracts from untreated cells (p<0.01), but without statistical differences in reactivity compared to extracts from a genetic SYNTHESIS mutant (Figure 3A). Consistent with the whole cell data, acid-released polysaccharides from C7 treated cells also had low orcinol reactivity, statistically no different than a SYNTHESIS mutant (UTI89 Δawg; Figure 3B). We tested if C7-treatment resulted in spontaneous release of polysaccharide into the media without proper anchoring into the outer membrane. LPS alterations have previously been shown to affect encapsulation [33]. However, concentrated ultrafiltrates of culture supernatants from C7-treated bacteria did not produce agglutination with antibody (data not shown). Furthermore, C7 treatment did not affect LPS abundance or migration on SDS PAGE gels (Figure S1; method described in Materials and References S1), indicating that lack of phage sensitivity and other phenotypes associated with C7 treatment are not due to defects in LPS. Together, these results suggest that C7 may be blocking an early step in capsule assembly, since weak agglutination and orcinol reactivity of whole cell sonicates of C7-treated cells indicates little to no intracellular accumulation of capsule and there was no detectable release of capsule upon acid treatment.

Whole cells and sonicates were used in agglutination and radial immuno diffusion assays using the H46 anti-K1 capsule polyclonal antiserum. Whereas wild-type and capsule synthesis mutants were positive and negative for agglutination, respectively, C7 treated UTI89 did not agglutinate and whole cell sonicates had only weak reactivity. We predicted that an inhibitor of K1 and K5 capsule biogenesis would be able to yield candidate small molecule inhibitors of Group 2 capsule biogenesis.

Table 2. Summary of capsule-specific and phage specific effects of selected lead compounds.

| NSC number | K1F phage inhibition | EV36/T7 phage lysis | MG/T7 phage lysis | Interpretation |
|------------|-----------------------|---------------------|------------------|----------------|
| 35505      | Yes                   | No                  | No               | Phage specific |
| 13028      | Yes                   | No                  | Yes              | Phage specific |
| 136469 (C7)| Yes                   | Yes                 | Yes              | Capsule specific |
| 5550       | Yes                   | Yes                 | Yes              | Capsule specific |
| 5159       | Yes                   | No                  | Not for 2 hr     | Phage specific |
| 661755     | Yes                   | No                  | Not for 2 hr     | Phage specific |
| 322921     | Yes                   | No                  | No               | Phage specific |
| 311153     | Yes                   | No                  | Not for 2 hr     | Phage specific |
| 311152     | Yes                   | No                  | Not for 3 hr     | Phage specific |

doi:10.1371/journal.pone.0011642.t002
C7 analogues as capsule inhibitors

In order to gain more insights into the important structural elements of C7, we also tested a limited series of structurally related compounds for inhibition of K1F phage lysis (Figure S2); however, none of the compounds tested was highly active. NSC201538 (2-(4-dimethylaminophenyl)benzo[g]quinoline-4-carboxylic acid) had limited activity, where the major substitution differentiating it from C7 was in the 4-dimethylaminophenyl substitution in place of the 4-phenyl-phenyl group present in C7, demonstrating the importance of the specific R-group for C7 activity.

Treatment increases C3 binding to UPEC K1 and serum sensitivity

As noted previously, polysaccharide capsules have known inhibition effects on complement binding to bacteria. We hypothesized that C7 treatment of UPEC K1 would result in increased binding by complement C3, initiating recruitment of the complement attack complex. UPEC K1 was treated with C7 or vehicle control and then incubated in normal human serum. C7 treatment produced a significant increase in C3 binding to the treated bacteria (~2-fold, Figure 4). These data suggest that C7 treatment renders the cells more susceptible to C3 binding, which in turn is known (specifically through C3b) to promote formation of the MAC complex and phagocytosis.

Given the increased binding of C3 to C7-treated cells, we anticipated that the chemically treated cells would have increased serum sensitivity similar to a genetic capsule mutant. Because of the established role of capsule in resistance by E. coli to serum killing, chemical capsule inhibition would be expected to sensitize encapsulated strains to serum exposure, thus attenuating infection and providing a useful therapeutic intervention. We tested whether exposure of the K1 encapsulated strain UTI89 to C7 increased its sensitivity to human serum in an in vitro assay with pooled human serum. After a 2 hour exposure of 10^5 CFU to 20% normal active human serum resulted, 35% of UPEC K1 remained viable. In contrast, the same serum exposure resulted in complete killing of the SYNTHESIS mutant (Figure 5). C7 treatment (100 μM) of UPEC K1 followed by the same serum exposure resulted in <1% of the bacteria remaining viable, statistically the same as for the genetic capsule mutant (no significant difference by Tukey’s Multiple Comparison Test). C7 treatment rendered the cells significantly more serum sensitive than untreated wild type (p<0.001). These results illustrate the potential of capsule biogenesis inhibitors to increase the susceptibility of UPEC to the innate immune response.

Table 3. Summary of agglutination assays.

| Strain          | Whole cell sonicates | Radial immunodiffusion assay |
|-----------------|----------------------|-----------------------------|
| K1              | +                    | +                           |
| K1 synthesis    | –                    | –                           |
| K1+100 μM C7    | weak                 | –                           |

Whole cells and sonicates were used in agglutination and radial immunodiffusion assays using the H46 anti-K1 capsule polyclonal antiserum. Whereas wild-type and capsule synthesis mutants were positive and negative for agglutination, respectively, C7 treated UTI89 did not agglutinate and whole cell sonicates had only weak reactivity.

doi:10.1371/journal.pone.0011642.t003

C7 is active on clinical E. coli isolates

To demonstrate that the effect of C7 is not limited to the prototypic laboratory UPEC strain UTI89, capsule inhibition by C7 was tested on a panel of clinical E. coli K1 isolates in the K1F phage sensitivity assay. These strains encompass isolates from pyelonephritis (4), recurrent UTIs (3), and single UTI cases (1). As seen in Figure 6, the majority of phage-sensitive isolates tested responded to C7 treatment by becoming insensitive to K1F phage, thus suggesting that capsule production was inhibited by the addition of C7 in these isolates, similar to the situation with strain UTI89. Two strains responded to C7 with only partial insensitivity to K1F, suggesting incomplete inhibition of capsule biogenesis. These results support the idea that C7 is active on a range of clinically relevant strains causing UTIs.
C7 is non-toxic to bladder epithelial cells

To further assess the therapeutic potential of C7, we next examined the potential toxicity of C7 using a widely used LDH release assay after treatment of the bladder epithelial cell line 5637 at several C7 concentrations up to 100 μM (4-fold over the IC50), as compared to 1% DMSO vehicle. No significant increase in LDH was measured at the maximum tested concentration of C7 compared to the vehicle control (Figure S3; methods described in Materials and References S1).

Discussion

E. coli is by-far the leading cause of community-acquired UTIs. On an annual basis, over 100 million UTIs occur throughout the
world with over 10 million infections in the US alone (per NIDDK data, [2]). Rising resistance rates have threatened the arsenal of antibiotics available for the treatment of community-acquired UTI. New therapeutics for UTI are in great demand. The most desirable chemotherapeutics may be those that attenuate an organism during an infection without altering commensal populations, so called anti-virulence agents [36,37].

UTI involved UPEC cycling between extracellular and intracellular environments. Bacteria initially adhere to the bladder epithelium, following which they may invade the epithelium, and in some cases, escape into the cytosol of the infected epithelial cells and amass into intracellular bacterial communities (IBCs). IBC confer resistance to lysis by neutrophils [38,39] and may similarly reduce susceptibility to antibiotic therapy [40]. Intracellular bacteria can emerge from IBCs and cAMP-regulated exocytic vesicles and reinitiate adherence and invasion events [41,42]. Early in the infection, TLR4 stimulation by LPS [43] results in a strong inflammatory response, neutrophil recruitment, and elaboration of antimicrobial peptides [44,45]. K capsule is important for UPEC survival in the urinary tract, promoting virulence at multiple steps of pathogenesis, including extracellular and intracellular stages of infection [46]. We recently demonstrated a novel role for the intracellular expression of a polysaccharide capsule as an aggregating factor in IBC formation [46]. Thus, inhibitors of K capsule biogenesis may attenuate the bacterium at multiple stages of infection.

Of the K type capsules, Groups 2 and 3 are overrepresented among the extraintestinal pathogenic E. coli including UTI, bloodstream and meningitis isolates. These capsular groups are uncommon among commensal E. coli, and therefore, it may be possible to selectively target the pathogenic organisms, particularly during active infection, while leaving commensal organisms unperturbed. In addition to avoiding dysbioses such as antibiotic-induced diarrhea caused by non-specific anti-microbial agents, anti-infectives that do not stress commensal microbial reservoirs may also lessen the emergence of drug resistance. Key components of the assembly and export of Group 2 and Group 3 capsules are highly conserved (such as KpsD3, KpsC, KpsU, KpsS, and KpsM), making it theoretically possible to identify small molecules that are capable of inhibiting the biosynthesis of a variety of capsules that differ significantly in composition and antigenicity. However, many of these same components are not well conserved among the other capsular types more commonly expressed by commensal strains.

By exploiting the features of capsule-specific phage, we devised an innovative, yet simple screen for small molecule inhibitors of K capsule biogenesis. To demonstrate the throughput and efficiency of the primary and secondary screens to identify capsule biogenesis inhibitors and eliminate false positive hits, we applied the screening procedure to a small collection of molecule libraries and identified one agent designated as “C7” that inhibits the production K1 and K5 capsules, unrelated polysaccharide types among the Group 2 capsules. C7 rendered clinical isolates of K1 and K5 encapsulated UPEC resistant to capsule-specific phage, decreased K1-antiserum agglutination, and shifted the carbohydrate profile of treated cells toward that of a genetic capsule synthesis mutant. Of biological significance, treatment of E. coli with C7 rendered the organism more susceptible to C3 binding and serum killing, again mirroring the phenotype of an unencapsulated genetic mutant. The IC50 of C7 between 12.5 and 25 μM suggests reasonable activity and a starting point for additional workarounds. The relative hydrophobicity of C7 imposes some limitations on its potential distribution and bioavailability if taken as a drug. Despite any potential limitations of C7, our results provide proof-of-concept that the screening algorithm may uncover additional novel small molecule inhibitors of capsule biogenesis by applying the screen to libraries several orders of magnitude larger in size.

In addition to their potential utility as therapeutic agents to combat infections, chemical inhibitors of capsule biogenesis may provide opportunities to further dissect the molecular processes involved in capsule biogenesis. Our current biochemical and immunological studies of the C7 effects on K1 capsule biogenesis have localized the inhibition to an early stage of capsule assembly, likely after sialic acid synthesis but prior to significant oligomerization of the monosaccharides. Our studies excluded other plausible mechanisms including alteration of LPS and failure to anchor fully polymerized polysaccharide to the bacterial surface. Additional studies will be required to improve our understanding of the molecular mechanisms and targets behind the action of C7. The genetic identity of the target of C7 would provide a better understanding of the capsule biosynthesis process and could greatly enhance the search for chemotherapeutics that could target highly conserved components of the capsule assembly machinery. This could lead to the attenuation of diverse encapsulated organisms with commonalities in capsule assembly. We anticipate that a large chemical library screen
currently underway that employs the primary and secondary screens described in this work will yield a variety of active, bioavailable, and synthetically amenable lead compounds to further build on this technology.

**Methods**

**Ethics statement**

Normal human serum samples were obtained under a protocol reviewed and approved by the Duke University Institutional Review Board. The pooled serum used in these studies was de-identified and anonymous.

**Bacterial strains, phage, and growth conditions**

All *E. coli* strains and phage used in the present study are listed in Table 4. Clinical *E. coli* isolates were obtained from Dr. Walt Stamm at the University of Washington. Unless indicated otherwise, bacteria were routinely grown at 37°C in Luria-Bertani medium (LB) with shaking at 250 rpm. Media were supplemented with antibiotics as needed at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml. LB was supplemented with 1% dimethyl sulfoxide (DMSO; Acros) with or without compound. Phage lysates were prepared from 5 ml cultures of UTI89 (for K1F phage), MG1655 (for ‘T7 phage) or DS17 (for K5 phage) and stored at 4°C over several drops of chloroform as described by [47].

**Chemicals**

Chemical compounds were obtained from the National Cancer Institute’s Developmental Therapeutics Program (DTP). Compounds were received in DMSO at a concentration of 10 mM and stored at −20°C in small aliquots protected from light.

**Screen to identify small molecular inhibitors of K1 capsule biosynthesis in UPEC**

Three ml overnight culture of K1 strain UTI89 grown in LB was diluted 1:500, and 100 μl per well was added to a 96-well plate (Whatman Uniplate). One microliter of each compound from the DTP small molecule library was placed in appropriate plate (Whatman Uniplate). One microliter of each compound prepared from 5 ml cultures of UTI89 (for K1F phage), MG1655 (for ‘T7 phage) or DS17 (for K5 phage) and stored at 4°C over several drops of chloroform as described by [47].

**Table 4. Bacteria and phage used in study.**

| Strain/phage | Description or relevant genotype | Reference |
|--------------|---------------------------------|-----------|
| **Bacterial strains** |                                  |           |
| UTI89        | K1 Escherichia coli cystitis isolate [50] |           |
| UTI89 Δneu   | Region II K1 capsule synthesis mutant [46] |           |
| UTI89 ΔkpsM  | Region III K1 capsule assembly mutant [51] |           |
| DS17         | Escherichia coli K5 pyelonephritis isolate |           |
| EV36         | K12/K1 hybrid produced by conjugation with an Hfr kps’ strain; K1 encapsulated, susceptible to K1 specific phage. [52] |           |
| **Phage**    |                                  |           |
| T7 phage (T7h) | Inhibited by K1 capsule [30] |           |
| K1F phage (K1Fh) | K1 capsule specific [26,27] |           |
| K5 phage (K5h) | K5 capsule specific [53] |           |

doi:10.1371/journal.pone.0011642.t004

**Capsule inhibition assay in test tubes**

Overnight bacterial cultures were diluted 1:100 into three ml of fresh LB supplemented with 100 μM of compound C7 or 1% vehicle control (DMSO). Cultures were grown at 37°C to an optical density OD\(_{600}\) of ~0.2 before addition of appropriate K1 capsule-specific phage or T7 phage (3 μl of lysate). Cultures were incubated further, and growth was monitored by optical density. The initial absorbance before infection was subtracted from the reading at the indicated time point after infection. The log of the absorbance values was then normalized to that of the culture with no phage added, and relative change was plotted. The average of triplicate replicates for a representative experiment was plotted with standard deviations. Each experiment was repeated at least twice with similar results to those shown.

**K1 capsule agglutination assay**

Three ml cultures were grown in the presence of 100 μM compound or vehicle to an optical density of ~0.8, and cells were harvested, washed with PBS, and resuspended in 0.5 ml of PBS. Fifty μl of cells and 5 μl of undiluted H46 horse anti-Group B *Neisseria meningitidis* capsule (antigenically identical to K1 capsule) polyclonal antiserum [49] were combined, and agglutination was monitored. This assay was repeated at least three times for each strain.

**Radial immunodiffusion assay**

PBS plates with 1% agarose were supplemented with 5% H46 antiserum. Cultures grown as for agglutination assays in the presence of 100 μM compound or vehicle were extensively sonicated to produce whole-cell lysates and added to wells created in plates (50 μl). Plates were incubated upright at 30°C for 48–72 hrs and were visually inspected for the formation of precipitin rings.

**Orcinol reaction for carbohydrates**

Three ml of cultures of the indicated strains were harvested at OD\(_{600}\) = 0.8. For whole-cell orcinol measurements, cells were washed once with PBS, resuspended in 1 ml of PBS, and sonicated. Five hundred microliters of each sonicate were then extracted with phenolchloroform, and 100 μl was used for labeling. For released material measurements, polysaccharides were separated from the cells by mild acid release (Tri-acetate pH 5.0 for 2 hr with shaking) followed by centrifugation to pellet the cells. Polysaccharides in the cell-free supernatants were then de-proteinated by phenolchloroform extraction. The aqueous material was concentrated to 50 μl using a YM-30 size exclusion filter.

For labeling, polysaccharides were then hydrolyzed with 0.1 M HCl for 5 minutes at 90°C in 50 μl of orcinol reagent [32]. Color change was measured by absorbance and expressed as the percentage of wild type levels. Each orcinol reaction was performed in duplicate with independent cultures, and the entire assay was repeated at least twice. A representative experiment is shown.
C3 binding assay

Human serum from at least two individuals was heat inactivated (HIS) at 55°C for 45 min or was maintained on ice (normal serum, NS). Cells were grown to OD900=1.0 and washed with PBS as described for the agglutination assay. Cells were then pelleted at 8000×g for 1 min and resuspended in DMEM (Sigma) +5% NS or HIS and were incubated at 37°C for 15 minutes. Bacteria were then pelleted and washed three times with PBS before resuspending in 500 μl of PBS. Three microliters were then spotted onto nitrocellulose membranes and allowed to air dry overnight. The membrane was blocked with 5% non-fat dry milk in TBS/0.1% Tween 20 (TBS-T) for 2 hrs, rinsed with TBS-T two times for 15 minutes and exposed to the primary C3 antibody (1:10,000 in 1% BSA/TBS-T, anti-human C3 developed, from Sigma). After washing two times with TBS-T, secondary anti-goat alkaline phosphatase antibody was applied at a 1:10,000 dilution. The immuno dot blot was developed using a colorimetric substrate (ImmunOx; MP Biochemicals), and densitometry was performed using Image J (NCBI) software.

Serum resistance assays

Overnight cultures of the indicated strains were diluted 1:100 into three ml of LB with 1% DMSO (final) or 100 μM C7. Cultures were grown for ~3 hrs with shaking until reaching an OD900 of 0.8. Bacterial cells were resuspended in PBS, diluted to 5–9×10^5 CFU/ml and incubated in serum-free RPMI (Sigma) supplemented with 20% human serum and 1% DMSO or 100 μM C7. Cells were incubated in RPMI/serum at 37°C for 2.0 hrs. Duplicate independent cultures for each strain were tested, and the ratio of NS/HIS CFU/ml is shown. Experiments were repeated at least two times.

Phage sensitivity assays for clinical E. coli strains

Clinical E. coli strains were screened for K1F and K5 phage sensitivity in 96-well trays. Briefly, a panel of clinical fecal, bloodstream, and urinary tract E. coli isolates from Walt Stamm at the University of Washington was arrayed into a 96-well plate and grown with vigorous shaking in LB in deep-well 96-well plates. Cultures were then diluted 1:100 into 100 μl of LB with 1% DMSO. The plate was sealed with breathable tape and incubated at 37°C with vigorous shaking for 2 hrs (OD900~0.1–0.2). Five microliters of a high-titer phage lysate were then added to each well. Growth was monitored spectrophotometrically every 1.5 hrs and “phage sensitive strains” were determined to be those strains for which absorbance decreased after 3.0 hrs of incubation. Each strain was tested in at least four independent plates. Isolates that were consistently lysed by addition of phage were then used in subsequent experiments to determine if addition of 100 μM C7 inhibited phage lysis using the same 96-well plate format. Inhibition of phage lysis by C7 was considered to be growth equivalent to uninfected parent strain after 3 hrs incubation post-infection, and this experiment was repeated at least 4 times with similar results. Relative change in OD was calculated as the log of the absorbance values and normalized to that of strain UTI89 in the same assay. The average of three independent cultures for each strain is shown.

Statistical analyses

Results were calculated as averages and standard deviations of the means using the Graph Pad Prism 4 software package (San Diego, CA). Nonparametric t-tests were used for statistical analysis of data and calculation of p-values using Graph Pad Prism 4 or Graph Pad online calculators. Significant differences were highlighted with a single asterisk when the P value is less than 0.05, with two asterisks when the P value is less than 0.01, and three asterisks when the P value is less than 0.001.

Supporting Information

Materials and References S1

Supplemental methods and references.
Found at: doi:10.1371/journal.pone.0011642.s001 (0.04 MB DOC)

Figure S1

C7 is non-toxic to bladder epithelial cells. LDH release assay after incubation of 5637 cells with vehicle (1% DMSO) or C7. Concentrations of C7 up to 100 μM did not significantly affect LDH release compared to vehicle control. Triton X detergent control represents maximum LDH release. Found at: doi:10.1371/journal.pone.0011642.s002 (0.16 MB TIF)

Figure S2

C7 treatment does not affect LPS profile. No significant difference was observed in LPS migration or accumulation with and without C7 treatment (Lane 1 vs. 2). The figure represents two independent gels with the same set of samples. Found at: doi:10.1371/journal.pone.0011642.s003 (3.89 MB TIF)

Figure S3

Compounds similar to C7 do not inhibit K1 capsule-dependent phage lysis. Panel A: Analogues of C7 tested as K1 capsule biogenesis inhibitors. Panel B: K1F phage sensitivity assays with 100 μM C7 and analogues. Found at: doi:10.1371/journal.pone.0011642.s004 (0.98 MB TIF)

Table S1

Similarity and identity of key proteins of group 2 capsule biogenesis in E. coli. UTI89 (K1, Group 2 capsule) proteins were compared to other E. coli Group 2 capsule homologues and minimum percent identity and similarity indicated. The Basic Local Alignment Search Tool (BLAST, NCBI) was used to compare key Group 2 capsule assembly proteins from the prototypic K1 strain UTI89 with sequenced E. coli genomes (taxonomic ID 562) with Group 2 capsule gene arrangement. The following sequenced Escherichia coli genomes were considered in the BLAST search: UTI89, SMS 3-5, ED1a, IAI39, APEC01, S88, 042, F11, SE15, BL21 (DE3), Nissle 1917, 101-1. Found at: doi:10.1371/journal.pone.0011642.s005 (0.03 MB DOC)

Acknowledgments

The authors thank Joseph St. Geme and Ravi Jhaveri for insightful and critical readings of the manuscript. Richard Silver kindly provided the horse Group B meningococcal antiserum (H46). The authors thank Dr. Ian Roberts for kindly providing the K5 bacteriophage used in these studies. We would like to also thank the Developmental Therapeutics Program at the National Cancer Institute for providing compounds.

Author Contributions

Conceived and designed the experiments: PCS. Performed the experiments: CCG PCS. Analyzed the data: CCG PCS. Contributed reagents/materials/analysis tools: PCS. Wrote the paper: CCG PCS.

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