Exploitation of Antibiotic Resistance as a Novel Drug Target: Development of a β-Lactamase-Activated Antibacterial Prodrug

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ABSTRACT: Expression of β-lactamase is the single most prevalent determinant of antibiotic resistance, rendering bacteria resistant to β-lactam antibiotics. In this article, we describe the development of an antibiotic prodrug that combines ciprofloxacin with a β-lactamase-cleavable motif. The prodrug is only bactericidal after activation by β-lactamase. Bactericidal activity comparable to ciprofloxacin is demonstrated against clinically relevant E. coli isolates expressing diverse β-lactamases; bactericidal activity was not observed in strains without β-lactamase. These findings demonstrate that it is possible to exploit antibiotic resistance to selectively target β-lactamase-producing bacteria using our prodrug approach, without adversely affecting bacteria that do not produce β-lactamase. This paves the way for selective targeting of drug-resistant pathogens without disrupting or selecting for resistance within the microbiota, reducing the rate of secondary infections and subsequent antibiotic use.

INTRODUCTION

Antimicrobial drug resistance is a global health emergency, threatening advances in many areas of medicine including surgery, cancer chemotherapy, organ transplantation, and survival of preterm infants.1,2 The most prevalent and important resistance determinant is the β-lactamase enzyme, which hydrolyzes members of the β-lactam class of antibiotic (e.g., penicillin, cephalosporins, and carbapenems) and thereby prevents engagement with their therapeutic targets the penicillin-binding proteins (PBPs).3,4 Of particular concern are the extended-spectrum β-lactamases (ESBLs) such as the CTX-M class, which are able to cleave a wide range of clinically relevant β-lactam antibiotics.5–7

Urinary tract infections (UTIs) are the most prevalent type of bacterial infection globally. These infections have a high rate of recurrence and can also lead to serious invasive infections such as sepsis, particularly in the elderly.8,9 E. coli is the most common causative organism (~75% cases), of which ~50% are resistant to β-lactam antibiotics due to β-lactamase expression.9,10 As a consequence of the high rate of β-lactam resistance in UTI pathogens, second-line, broad-spectrum antibiotics such as ciprofloxacin are increasingly used therapeutically.11,12 Unfortunately, these broad-spectrum antibiotics are associated with disruption to the beneficial bacteria that colonize the gastrointestinal tract and other surfaces, known as the microbiota.13–17 This disruption can lead to serious secondary infections by antibiotic-resistant bacteria such as Clastidium difficile or fungi such as Candida albicans, leading to colitis and thrush, respectively.15,18 This is because antibiotics target conserved processes in bacteria such as cell wall, protein, DNA or RNA biosynthesis, which not only occur in the pathogens that cause infection but also in the members of the microbiota.19,20

An additional complication associated with some second-line therapeutics such as ciprofloxacin is host toxicity. Ciprofloxacin holds two black box warnings, one for increased risk of tendinitis and tendon rupture and one for exacerbation of muscle weakness in myasthenia gravis sufferers.21 Additionally, in 2015, the FDA officially recognized fluoroquinolone-associated disability (FQAD) as a syndrome. FQAD describes a range of disabling and potentially permanent side effects including disturbances of tendons, joints, muscles, nerves, the nervous system, and induction of type 2 diabetes.22,23 As a result, strategies with the potential to mitigate host toxicity by reducing exposure to ciprofloxacin are needed.

Given the drawbacks associated with broad-spectrum antibiotics, efforts have been made to limit their use.11,23–25

Received: December 7, 2018
Published: April 22, 2019

DOI: 10.1021/acs.jmedchem.8b01923
However, these efforts have had limited success with usage rates increasing globally, particularly in low- and middle-income countries. In part, this is due to a lack of access to fast and efficient diagnostic techniques and the need to respond quickly to serious bacterial infections with effective and cost-efficient treatment regimens that target a wide range of different bacterial pathogens. Consequently, there is, therefore, a pressing need to develop new therapeutics that kill a broad range of different pathogens without damaging the host microbiota.

Since -lactamase enzymes are not found in mammalian cells, we hypothesized that we could exploit this enzyme as a novel antibacterial target. Furthermore, -lactamase expression is prevalent among UTI pathogens, which can both colonize the gut and cause infection of the GU tract. Consequently, -lactamase expression is prevalent among UTI pathogens, which can both colonize the gut and cause infection of the GU tract.8,10 Consequently, this represents an opportunity to selectively target disease-causing bacteria without causing significant disruption to the microbiota or select for drug resistance as has been reported for broad-spectrum antibiotics such as ciprofloxacin. Therefore, the aim of this work was to develop a small molecule antibacterial agent that is selectively active against bacteria that express -lactamase. To do this, we employed a prodrug strategy that utilized a -lactam cleavable motif linked to the broad-spectrum antibiotic ciprofloxacin.

In support of our approach, the use of -lactams as prodrug modifiers in antibody-directed enzyme prodrug therapy approaches has been explored in disease areas such as cancer (1–3, Figure 1).31−35 Additionally, -lactam–fluoroquinolone conjugates have been proposed as a co-drug strategy to treat bacterial infections (4 and 5, Figure 1).36−39 However, our approach is different in that it is designed to selectively deliver a broad-spectrum, bactericidal antibiotic to only bacteria that express -lactamase, while having minimal effect on bacteria that do not express the resistance determinant. By contrast, previous dual activity co-drug approaches were designed to have broad-spectrum activity against both drug-sensitive bacteria and those that express -lactamase.

Herein we describe the design and development of the prodrug, including optimization of the -lactam motif to reduce the antibacterial activity of the intact molecule and increase the efficiency of -lactamase mediated ciprofloxacin release. This is, to our knowledge, the first example of a -lactam–fluoroquinolone prodrug with selective activity against drug-resistant bacteria.

## RESULTS AND DISCUSSION

### Prodrug Design

In order to create a prodrug molecule that is selectively activated in -lactamase producing bacteria, it was important to select a -lactamase cleavable motif, linkage strategy, and active antibiotic that gave a stable nonbactericidal drug-resistant bacteria.

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The cephalosporin class of -lactams are efficiently hydrolyzed by -lactamases and have been previously employed as a prodrug motif as cleavage of the -lactam ring is associated with the loss of the functional group at the 3′-position (Figure 2A).40 In addition, the chemistry associated with changing the 3′-substituent of cefazolin is well-established and a wide variety of substituents at the C-3′ position are tolerated by -lactamases.37,41,42 Consequently, a cephalosporin core was selected as the -lactam component. To achieve the desired selectivity profile, ciprofloxacin was attached via the carboxylic acid to give the 3′-position (Figure 2B). Derivatization of the carboxylic acid group of fluoroquinolone antibiotics is associated with a significant decrease in antibacterial activity due to a decreased ability to bind to bacterial DNA–enzyme complexes.33 While this choice of attachment site was selected to remove the ciprofloxacin activity from the intact prodrug, it remained likely that the

![Figure 1. Selected representative examples of cephalosporin prodrugs (PROTAX 1, 2, and BMY-46633 3) and co-drugs (MCO 4 and Ro 23-9424 5).](image-url)
prodrug molecule would retain antibacterial activity as a result of the ability of the cephem portion of the molecule to interact with PBPs. Therefore, to further increase selectivity, it was essential to undertake a program of optimization of the \(\beta\)-lactam motif to reduce PBP activity and increase or maintain \(\beta\)-lactamase activity. Initial optimization was performed on the cefalosporin portion of the prodrug to enable the rapid generation of analogues and evaluation of biological activity. The cefalosporin analogues with the most desirable activity profile were then selected for preparation as the full prodrug.

**\(\beta\)-Lactam Analogue Preparation and Biological Evaluation.** Analysis of the literature identified the amide functionality at C-7 of the cephem ring as central to PBP and \(\beta\)-lactamase activity,\(^{44 - 51}\) and therefore structural changes at this position provided the initial focus of investigation. By use of cephalothin 7 (Table 1) as the starting point, analogues were prepared to explore bioisosteric replacement,\(^{52,53}\) functionalities present in early generation \(\beta\)-lactam antibiotics, and to probe steric and electronic tolerance.\(^{54,55}\) All compounds were synthesized according to the previously reported methods (Figure S1 in Supporting Information).\(^{55,56}\)

Antibacterial activity was assessed by determining the minimal concentration required to inhibit bacterial growth, known as the minimal inhibitory concentration (MIC), against the *E. coli* strain DH5\(\alpha\)\(^{57}\) expressing the ESBL TEM-116. The susceptibility to \(\beta\)-lactamase mediated hydrolysis was assessed by determining the physiological efficiency (\(k_{\text{cat}}/K_m\)) of hydrolysis by recombinant AmpC protein.\(^{3,58,59}\)

For all compounds (Table 1), a higher MIC value was determined for the *E. coli* strain expressing TEM-116 than the strain not expressing \(\beta\)-lactamase, indicating hydrolytic activity by the \(\beta\)-lactamase. Introduction of a substituent to the thiophene ring (8) or switching from a C-2 to a C-3 substitution (9) gave a modest increase in MIC values and a small decrease in \(k_{\text{cat}}/K_m\) compared to cephalothin 7. Although no measurable MIC value could be determined for any of the phenyl analogues (20–23), this was accompanied by a >3-fold decrease in \(k_{\text{cat}}/K_m\). In general, a quaternary carbon (20–24) or tertiary carbon (10 and 25) at the \(\alpha\)-position relative to the amide carbonyl was not well tolerated by AmpC. This finding is consistent with prior reports and has previously been exploited to reduce \(\beta\)-lactamase activity in the development of later-generation \(\beta\)-lactams. Compounds containing straight-chain aliphatic groups (26–28) retained some antibacterial activity; an increase in \(k_{\text{cat}}/K_m\) was observed with increasing chain length.

Examination of the tested analogues (Table 1) immediately revealed the importance of bulky benzylic substituents (11–19). Thiophene rings are frequently used as a bioisosteric replacement for a phenyl groups, and it is therefore perhaps unsurprising that there was only a modest 4-fold increase in MIC value against *E. coli* DH5\(\alpha\) and a slight decrease in \(k_{\text{cat}}/K_m\) for 11 compared to cephalothin.\(^{52,53}\) However, introduction of substituents at the para-position (12–17) gave a further 2- to 4-fold increase in MIC against *E. coli* DH5\(\alpha\) compared to unsubstituted benzyl 11. Substitution at the para-
position also affected hydrolysis by AmpC with the following order of activity observed: F < Me = H < Cl = Br. Movement of the methyl substituent from the para- (12) to the meta-position (18) gave a 5-fold increase in $k_{cat}/K_m$ and a 3-fold increase compared to cephalothin. High $k_{cat}/K_m$ values were determined for bisaryl 16 and the para- and meta-substituted biphenyl ethers 17 and 19 (3.99 ± 0.88, 7.33 ± 1.72, and 31.14 ± 2.67, respectively). In addition, no measurable MIC values could be determined for 16, 17, or 19. This led us to question if the results were indicative of no antibacterial activity or simply a result of increased efflux activity out of, or a lack of permeability into, the bacterial cell.

**β-Lactamase Hydrolytic Activity in Whole-Cell NMR Assay.** To address the question of compound permeability/efflux, a whole-cell β-lactamase hydrolysis assay was used to detect the penetration of compounds into the periplasm.60,61 Hydrolytic decomposition of β-lactam rings is associated with changes in $^1$H NMR signals, which can be detected using whole bacterial cells in real time by $^1$H NMR spectroscopy (Figure S2). As hydrolysis occurred within the bacterial periplasm, only compounds with sufficient intracellular accumulation were hydrolyzed. Compounds 16 and 17 were selected as representative examples of high lipophilicity compounds with no measurable antibacterial activity and moderate in vitro β-lactamase hydrolysis. We evaluated the hydrolysis of bisaryl 16, biaryl ether 17, and cephalothin 7 in DH5α ± TEM-116 (Table 2). After 90 min, 16 was 69% hydrolyzed in DH5α ± TEM-116 compared to 14% hydrolyzed in DH5α ± TEM-116 and 17 was 53% hydrolyzed in DH5α ± TEM-116 compared to 13% hydrolyzed in DH5α ± TEM-116. These results indicated a high degree of in vivo β-lactamase mediated hydrolysis and that 16 and 17 accumulated in the bacterial cell. We therefore concluded that the lack of antibacterial activity of this compound against E. coli DH5α without β-lactamase was due to an absence of PBP engagement and not due to poor permeability or efflux activity.

**Biological Evaluation in Uropathogenic E. coli.** Initial assessment of compound activity was performed in the laboratory E. coli strain DH5α. To assess the activity of the β-lactams against a clinically relevant pathogenic strain of E. coli, we selected the uropathogenic strain CFT073. This bacterium was isolated from the blood of a patient with acute pyelonephritis, is devoid of all virulence plasmids commonly associated with uropathogenic strains, and proved tractable for genetic manipulation.62,63 The plasmid pSU18, without the coding sequence for β-lactamase (referred to here as pEMP) or encoding for the β-lactamase CTX-M-1, was introduced into CFT073, enabling comparison of compound activity in CFT073 and CFT073 + pSU18 ± β-lactamase. The primary β-lactam used in this work was CTX-M-1 because CTX-M enzymes are the most prevalent β-lactamases among enterobacteria such as E. coli. As part of a class of extended-spectrum β-lactamases (ESBL) it confers resistance to most β-lactam antibiotics, with the exception of carbapenems.64

In the first instance, MIC values were determined for selected compounds against CFT073 + pSU18 ± CTX-M-1 (Table 3). For all the compounds tested, the MIC values for CFT073 + pSU18 were within 2-fold of those determined against DH5α. Next the hydrolytic activity of these compounds was assessed in the whole cell NMR assay (Table 3). For the majority of the compounds tested, a low level of hydrolysis, <20% after 60 min, was detected. However, levels of hydrolysis comparable to that observed for cephalothin (68%) were observed only for 24 and 26 (64% and 67%, respectively).

A clear feature of the SAR was that CTX-M-1 mediated hydrolytic activity in whole CFT073 cells correlated with lipophilicity. Plotting the calculated log P (cLogP) values for compounds against the log of percentage hydrolysis revealed that moderate–high levels of hydrolysis (>30%) were only observed for compounds with cLogP values below 0.1 (Figure 3). Linear regression analysis revealed moderate correlation ($R^2 = 0.60$), depicting the degree of hydrolysis reflecting both cellular penetration and β-lactamase activity, which are both sensitive to compound lipophilicity.

Interestingly, compounds 16 and 17 were hydrolyzed rapidly (69% and 54% after 90 min, respectively) in DH5α expressing the TEM-116 β-lactamase (Table 2) but only 4% hydrolyzed after 60 min in CFT073 expressing the CTX-M-1 β-lactamase. We hypothesized that the low level of hydrolytic activity observed for many of the compounds could be a result of poor intracellular accumulation in CFT073 E. coli since clinical isolates often have reduced permeability to antibiotics.64 To test this hypothesis, hydrolysis in DH5α expressing CTX-M-1 was determined for cephalothin 7, 16, and 17 (Table 2). After 60 min complete hydrolysis for cephalothin 7 and 16 and 94% hydrolysis for 17 were observed, suggesting that the low level of hydrolysis observed in CFT073 + CTX-M-1 was not due to the inability of CTX-M-1 to hydrolyze this chemotype. Instead it is likely that due to poor membrane permeability or increased efflux activity, lipophilic analogues were unable to engage with CTX-M-1 in CFT073.

For compounds with low hydrolytic activity in the whole cell NMR assay with CFT073 we were unable to discern if a high MIC value in the absence of CTX-M-1 truly reflected a lack of antibacterial activity or a lack of permeability/high efflux activity. Therefore, compound 26, with its high MIC value in both CFT073 ± CTX-M-1 (≥400 μM) and high hydrolytic activity in whole CFT073 cells (67% after 60 min), was selected for incorporation into the full prodrug molecule. Compound 24, which also possessed a high MIC value in both CFT073 ± CTX-M-1 (≥400 μM) and high hydrolytic activity in whole CFT073 cells (64% after 60 min), was not progressed at this time as we wished to avoid the potential for toxicity problems arising from the furan ring, which has been identified as common toxicophore due to metabolic instability.65

**Prodrug Preparation.** Preparation of the prodrug derived from compound 26 required coupling of an activated 26 derivative, iodocephalosporin 30, to ciprofloxacin derivative 33 (Scheme 1). The iodocephalosporin 30 was prepared in three steps from commercially available 7-aminoccephalosporanic

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**Table 2. Percentage Hydrolysis of Cephalothin (Ceph) 7 and Compounds 16 and 17 by DH5α Cells ± β-Lactamase in Whole-Cell NMR Hydrolysis Assay**

<table>
<thead>
<tr>
<th>compd</th>
<th>conc (μM)</th>
<th>incubation time (min)</th>
<th>strain</th>
<th>% hydrolysis by NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceph 7</td>
<td>50</td>
<td>60</td>
<td>DH5α ± TEM-116</td>
<td>61</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>90</td>
<td>14</td>
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<tr>
<td>17</td>
<td>100</td>
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<tr>
<td>Ceph 7</td>
<td>100</td>
<td>60</td>
<td>DH5α ± CTX-M-1</td>
<td>0</td>
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<tr>
<td>16</td>
<td>100</td>
<td>60</td>
<td>0</td>
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<td>17</td>
<td>100</td>
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4414
acid (7-ACA). First, 7-ACA was reacted with acetic anhydride to give N-acetyl-
26.55 Protection of the carboxylic acid as the tert-butyl ester was then performed using tert-butyl 2,2,2-
trichloroacetimidate (TBTA) enabling formation of the tert-butyl ester in the absence of base, which has previously
been reported to be associated with isomerization from the Δ3-cephem to the biologically inactive Δ2-cephem.67
Iodination at the 3′-position with TMSI gave the activated iodocephalosporin ready for coupling.36 The cipro-
32. The carboxylic acid was then converted to the sodium salt of the corresponding sodium salt
33.70 Coupling of compounds 30 and 33 was performed in 3:1 1,4-dioxane/DMF to give the protected cephalosporin−ciprofloxacine conjugate.36,67
Finally, global deprotection with TFA to remove the BOC and tert-butyl ester afforded the final prodrug.71 Synthesis
35 was achieved in seven steps from commercially available materials without the requirement for toxic metal reagents.

**In Vitro DNA Gyrase Activity.** Members of the fluoroquinolone antibiotic family, including ciprofloxacine,
target the type II topoisomerase enzymes, DNA gyrase, and topoisomerase IV. Inhibition of these enzymes results in the
arrest of DNA replication and transcription preventing bacterial cell growth.43,72 Having successfully prepared prodrug
35, we moved to testing our hypothesis that the intact prodrug would not inhibit DNA gyrase or topoisomerase IV but β-
lactamase-triggered hydrolysis would result in the release of free ciprofloxacine capable of engaging these targets. To test this
hypothesis, we evaluated the ability of prodrug 35 and ciprofloxacine to inhibit recombinant DNA gyrase enzyme activity in the absence and presence of the purified recombinant β-lactamase CTX-M-15 (Figure 4). Compounds
were incubated with relaxed pBR322 plasmid DNA with and without recombinant CTX-M-15 and recombinant DNA
gyrase. As predicted, inhibition of DNA gyrase by prodrug 35 was not observed in the absence of CTX-M-15. However,
in the presence of CTX-M-15, 1 μM 35 was capable of reducing DNA gyrase activity by >50%. Ciprofloxacine activity was not a
35 was determined for cephalothin 7. As expected, the MIC value determined for ciprofloxacine was consistent across all tested strains at 31 nM. The MIC
determined for prodrug 35 in *E. coli* CFT073 WT and
expressing empty plasmid (pEMP) was 310 nM, representing a 10-fold decrease in activity compared to ciprofloxacin in the absence of β-lactamase. By contrast to bacteria without β-lactamase, the MIC value for E. coli CFT073 strains expressing CTX-M-1, NDM1, or KPC, only 2-fold higher than ciprofloxacin. These data demonstrate efficient and selective β-lactamase mediated prodrug cleavage and active antibiotic release, resulting in arrest of bacterial cell growth at concentrations comparable to that with free ciprofloxacin.

The activity of prodrug 35 compared to ciprofloxacin was profiled further in six independently isolated uropathogenic E. coli clinical isolates expressing the CTX-M-15 β-lactamase, which were obtained from Charing Cross Hospital, Imperial College NHS Trust. Three of the strains were ciprofloxacin sensitive (EC11, EC16, and EC17), and three were ciprofloxacin resistant (EC12, EC13, and EC19) as determined by diagnostic susceptibility testing. Activity of prodrug 35 was confirmed against the three ciprofloxacin sensitive bacterial strains, while no arrest in bacterial growth was observed for either ciprofloxacin 31 or prodrug 35 for strain EC12, EC13, or EC19 (Figure 6 and Table S1). These results demonstrate that the antibacterial activity of 35 observed in β-lactamase expressing strains is mediated through liberated ciprofloxacin and provide evidence for the clinical utility of 35. The gut microbiota includes both Gram-negative bacteria such as E. coli and Gram-positive organisms such as E. faecalis. Since we had shown that 35 was inactive against E. coli, we decided to further examine the potential clinical value of the prodrug by testing its activity against two representative E. faecalis strains that did not express β-lactamase. Prodrug 35 showed reduced activity compared to ciprofloxacin, indicating that our approach could minimize undesirable damage to the microbiota caused by fluoroquinolones (Figure S3). We also assessed the activity of 35 against CFT073 pEMP or pCTX-M-1 in the presence of human serum, which can modulate drug activity via protein-binding and also contains esterases that have the potential to activate the prodrug by cleaving the ester linkage. However, data from MIC assays performed in the presence of human serum (Figure S4) were equivalent to those obtained in the absence of serum (Figure 5). Combined, these

Figure 4. Activity of prodrug 35 and ciprofloxacin 31 against recombinant DNA gyrase ± CTX-M-15. (A) DNA was separated by agarose gel electrophoresis with 2 log DNA ladder: oc, open circle DNA; rel, relaxed DNA; sc, supercoiled DNA. (B) Quantification of gel bands corresponding to supercoiled DNA and normalized to no gyrase and gyrase only activity (ImageJ 1.52a): Gyr, DNA gyrase; cip, ciprofloxacin 31; PD, prodrug 35; CTX, CTX-M-15. Error bars represent SEM (n = 4); prodrug vs prodrug + CTX-M-15 was analyzed by unpaired t-test, p = 0.0004 (GraphPad Prism 7.03).

“Reagents and conditions: (i) acetic anhydride, NaHCO3, H2O, acetone, 0 °C, 30 min; (ii) TBTA, DCM, 60 °C, 16 h; (iii) TMSI, DCM, rt, 2 h; (iv) Boc2O, 1 M NaOH, THF, rt, 16 h; (v) 0.1 M NaOH, MeOH, rt, 30 min; (vi) 3:1 1,4-dioxane/DMF, rt, 4 h; (vii) 1:1 TFA/DCM, anisole, 0 °C to rt.

Scheme 1. Synthesis of Prodrug 35
findings provided further confidence in the selectivity of prodrug 35 and its stability in the host environment.

**Selective Bactericidal Activity against β-Lactamase Expressing Bacteria.** Finally, the ability of prodrug 35 to kill bacteria rather than arrest growth was evaluated. Survival of *E. coli* CFT073 pEMP or pCTX-M-1 with no treatment or exposed to ciprofloxacin 31 or prodrug 35 was determined over time by CFU counts (Figure 7). After 6 h incubation with prodrug 35 there was >100-fold greater killing of *E. coli* expressing CTX-M-1, compared with bacteria that did not express the enzyme. Killing activity of 35 in *E. coli* expressing CTX-M-1 was almost identical to free ciprofloxacin, while growth comparable to no treatment controls was detected for CFT073 expressing empty plasmid incubated with 35. These findings demonstrate that it is possible to selectively kill β-lactamase-producing bacteria using our prodrug approach,
lactamase. Though modifications have been designed, synthesized, and evaluated for biological activity, a program of optimization was successfully undertaken to reduce the antibacterial activity of the intact prodrug without adversely affecting bacteria that do not produce β-lactamase.

**CONCLUSIONS**

A novel cephalosporin–fluoroquinolone antibiotic prodrug has been designed, synthesized, and evaluated for biological activity. A program of optimization was successfully undertaken to reduce the antibacterial activity of the intact prodrug through modification to the cephalosporin component. Prodrug 35 exhibits similar growth inhibitory activity to ciprofloxacin against uropathogenic *E. coli* expressing the diverse ESBLs CTX-M-1, NDM-1, and KPC but little activity against strains that did not express β-lactamases. The selectively observed for bactericidal activity was even greater, with prodrug 35 killing β-lactamase expressing bacteria at the same rate as free ciprofloxacin while not affecting the growth of bacteria that did not express β-lactamases.

Overall, the activity of prodrug 35 is consistent with (1) permeability to pathogenic Gram-negative bacteria, (2) a low-level of antibacterial activity for the intact prodrug, (3) β-lactamase mediated intracellular release of ciprofloxacin upon cleavage of the cephalosporin, and (4) activation of the prodrug by a broad range of β-lactamases.

Together, these studies demonstrate that our prodrug approach can harness resistance as a therapeutic opportunity to selectively kill antibiotic-resistant bacteria. Since fluoroquinolones are a clinically useful, broad-spectrum antibiotic, we envisage that increasing the selectivity profile will have two major advantages. First, increased selectivity of fluoroquinolones will enable maintenance of the microbiota leading to reduced secondary infection rate and subsequent antibiotic use. Second, there is a decreased side-effect profile due to minimized exposure of host cells to fluoroquinolone antibiotic.

The focus of this work was uropathogenic *E. coli* (UPEC), which is a major cause of UTIs and frequently expresses β-lactamase. Our approach is expected to result in high survival of *CFT073* pEMP (open circle) and pCTX (filled circle) with no treatment (green), ciprofloxacin 31 (78 nM) (red), or prodrug 35 (78 nM) (blue): Cipro, ciprofloxacin; PD, prodrug 35.

Figure 7. Survival of CFT073 pEMP (open circle) and pCTX (filled circle) with no treatment (green), ciprofloxacin 31 (78 nM) (red), or prodrug 35 (78 nM) (blue): Cipro, ciprofloxacin; PD, prodrug 35.

Experimental Procedures (Chemistry). Unless otherwise stated, reactions were conducted in oven-dried glassware under an atmosphere of argon using anhydrous solvents. All commercially obtained reagents and solvents were used as received. TLC analysis was performed on precoated aluminum sheets of silica (60 F254 nm, Merck) and visualized using short-wave UV light. Column chromatography was also performed on an Isolera Spektra Four purification system using Biotage Flash silica cartridges (SNAP KPSil, SNAP Ultra, or SNAP KP-C18-HS).

**EXPERIMENTAL SECTION**

Experimental Procedures (Chemistry). Unless otherwise stated, reactions were conducted in oven-dried glassware under an atmosphere of argon using anhydrous solvents. All commercially obtained reagents and solvents were used as received. TLC analysis was performed on precoated aluminum sheets of silica (60 F254 nm, Merck) and visualized using short-wave UV light. Column chromatography was also performed on an Isolera Spektra Four purification system using Biotage Flash silica cartridges (SNAP KPSil, SNAP Ultra, or SNAP KP-C18-HS).
Method B. 7-Aminocarboxaldehyde (1 equiv) and acid chloride (2 equiv) were dissolved in EtOAc and heated to reflux for 30 min. After cooling to room temperature, aniline (1.3–3 equiv) was added and stirred for 1 h before the reaction mixture was diluted with 3% NaHCO₃ (aq). The aqueous layer was separated and the organic layer washed with 3% NaHCO₃ (aq) (×2). The aqueous layers were combined, washed with EtOAc, and acidified to pH 2 with 1 M HCl. The desired product was isolated as described.

Method C. Carboxylic acid (1 equiv) was dissolved in DCM and oxalyl chloride (1.2 equiv) added followed by DMF (1 drop) and the reaction stirred for 16 h. The solvent was removed under reduced pressure to afford the acyl chloride, which was used without further purification.

Preparation of Compounds. (6R,7R)-3-(Acetoxy)methyl-7-(2-(4-bromomethoxy-2-yl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (8). 2-(4-Bromothiophen-2-yl)acetic acid (203 mg, 0.92 mmol), N-(3-dimethylamino-propyl)-N′-ethylcarbodiimide hydrochloride (193 mg, 1.01 mmol), and 7-aminocarboxaldehyde (250 mg, 0.92 mmol) were suspended in DMF (8 mL) and stirred at room temperature for 48 h. The resulting mixture was filtered and the filtrate diluted with H₂O and extracted with EtOAc (×3). The organic extracts were combined, washed with 1 M LiCl (aq) and brine, and dried over Na₂SO₄. Solvent was removed under reduced pressure and the resulting oil triturated with Et₂O. The precipitate was collected by vacuum filtration and washed with DCM to afford the product as a beige amorphous solid (36 mg, 8%). IR (solid): νₑₒₓ 3273, 3101, 2837, 1774, 1748, 1707, 1662, 1539, 1233 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ 9.15 (d, J = 8.1 Hz, 1H), 7.51 (d, J = 1.2 Hz, 1H), 6.93 (s, 1H), 5.68−5.59 (m, 1H), 5.06 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.6 Hz, 1H), 4.70 (d, J = 12.6 Hz, 1H), 3.78 (s, J = 2.6 Hz, 2H), 3.58 (d, J = 18.0 Hz, 1H), 3.42 (d, J = 18.7 Hz, 1H), 2.02 (s, 3H). ¹³C NMR (100 MHz, DMSO-d₆) δ 170.3, 169.4, 162.8, 139.1, 128.8, 125.2, 107.7, 59.0, 57.2, 35.6, 25.4, 20.6. HRMS (ESI)¹: calc for C₁₆H₁₇BrN₂O₆S_2 (M+H)⁺ 496.9453, found 496.9479.

(6R,7R)-3-(Acetoxy)methyl-8-oxo-7-(2-thiophen-3-yl)-acetylamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (9). 3-Thiophenecetic acid (104 mg, 0.74 mmol), oxalyl chloride (76 µL, 0.88 mmol), and DMF (1 drop) were reacted in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocarboxaldehyde (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 µL) according to method B. The aqueous layer was extracted with DCM (×3), and the organic layers were combined, dried over Na₂SO₄, and evaporated. The resulting solid was triturated with ice-cold DCM to afford the product as an off-white amorphous solid (48 mg, 33%). IR (solid): νₑₒₓ 3284, 1751, 1730, 1651, 1621, 1536, 1241 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ 8.95 (d, J = 8.3 Hz, 1H), 7.46 (dd, J = 4.9, 3.0 Hz, 1H), 7.26 (dd, J = 2.9, 1.0 Hz, 1H), 7.03 (dd, J = 4.9, 1.2 Hz, 1H), 5.46 (dd, J = 8.3, 4.8 Hz, 1H), 4.99 (d, J = 11.9 Hz, 1H), 4.93 (d, J = 4.8 Hz, 1H), 4.73 (d, J = 11.9 Hz, 1H), 3.61−3.49 (m, 2H), 3.45 (d, J = 17.2 Hz, 1H), 3.19 (d, J = 17.3 Hz, 1H), 2.00 (s, 3H). ¹³C NMR (100 MHz, DMSO-d₆) δ 170.6, 170.5, 163.3, 153.8, 153.5, 128.6, 125.7, 122.3, 64.7, 58.5, 57.2, 36.4, 25.1, 20.8. HRMS (ESI)¹: calc for C₁₆H₁₅N₂O₆S (M + H)⁺ 405.1119, found 405.1119.

(6R,7R)-3-(Acetoxy)methyl-7-(2-(4-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (13). 4-Fluorophenylacetamide lactyl chloride (98 µL, 0.74 mmol) and 7-aminocarboxaldehyde (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 µL) according to method B. The resulting mixture was cooled to 4 °C and the precipitate collected by vacuum filtration and washed with ice-cold DCM to afford the product as an amorphous solid (61 mg, 41%). IR (solid): νₑₒₓ 3273, 1763, 1736, 1659, 1532, 1215 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ 13.67 (br s, 1H), 9.10 (d, J = 8.2 Hz, 1H), 7.30 (dd, J = 8.4, 5.6 Hz, 2H), 7.12 (app t, J = 8.8 Hz, 2H), 5.67 (dd, J = 4.8, 1.8 Hz, 1H), 5.08 (d, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.69 (d, J = 12.8 Hz, 1H), 3.62 (d, J = 18.4 Hz, 1H), 3.54−3.41 (m, 2H), 2.26 (s, 3H), 2.03 (s, 3H). ¹³C NMR (100 MHz, DMSO-d₆) δ 171.1, 170.4, 164.8, 162.8, 135.5, 132.7, 128.9, 128.8, 62.7, 59.1, 57.4, 41.2, 25.5, 20.6, 20.7. HRMS (ESI)¹: calc for C₁₉H₁₃FN₂O₅S (M + H)⁺ 409.0870, found 409.0864.

(6R,7R)-3-(Acetoxy)methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (14). Hexanoyl chloride (139 mg, 0.74 mmol) and 7-aminocarboxaldehyde (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (75 µL) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold DCM to afford the product as a cream amorphous solid (104 mg, 67%). IR (solid): νₑₒₓ 3265, 3056, 1778, 1748, 1707, 1643, 1536, 1223 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ 13.69 (br s, 1H), 9.13 (d, J = 8.2 Hz, 1H), 7.36 (d, J = 8.5 Hz, 2H), 7.26 (d, J = 8.5 Hz, 2H), 6.05 (s, J = 4.8 Hz, 1H), 5.00 (d, J = 12.8 Hz, 1H), 4.68 (d, J = 12.8 Hz, 1H), 3.65−3.45 (m, 4H), 2.03 (s, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 170.6, 170.2, 164.7, 162.8, 135.8, 129.0, 128.2, 126.4, 125.2, 62.7, 59.1, 57.4, 41.6, 25.5, 20.6. HRMS (ESI)¹: calc for C₁₉H₁₃N₂O₅S (M + H)⁺ 447.0394, found 447.0414.
(6R,7R)-3-(Acetoxymethyl)-7-(2-(4-bromophenyl)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (15). 3-Bromophenylacetyl chloride (171 mg, 0.74 mmol) and DMF (1 drop) were reacted in DCM (3 mL) according to method B. The aqueous layer was extracted with DCM (5 mL) prior to the addition of aniline (0.37 mmol) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold DCM to afford the product as a cream amorphous solid (112 mg, 65%). IR (solid): 3280, 1774, 1730, 1655, 1532, 1223 cm⁻¹. HRMS (ESI+): calcd for C₁₈H₁₈BrN₂O₆S (M + H)⁺ 469.0093, found 469.0076.

(6R,7R)-7-(1-Biphenyl)-4-yl)acetamido)-3-(acetoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (16). 4-Biphenylacetic acid (170 mg, 0.736 mmol), oxalyl chloride (76 μL, 0.88 mmol), and DMF (1 drop) were reacted in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (100 mg, 0.37 mmol) were reacted in DCM (5 mL) according to method B. The aqueous layer was extracted with DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (0.37 mmol) were reacted in DCM (5 mL) prior to the addition of aniline (75 μL) according to method B. The aqueous layer was extracted with DCM (x3), and the organic layers were combined, dried over Na₂SO₄, and evaporated. The resulting solid was precipitated from hot DCM to afford the product as a white amorphous solid (75 mg, 55%). IR (solid): 3254, 1774, 1752, 1710, 1651, 1520, 1223 cm⁻¹. HRMS (ESI+): calcd for C₁₉H₂₁N₂O₆S (M + H)⁺ 483.1226, found 483.1233.

(6R,7R)-3-(Acetoxymethyl)-7-(2-(3-phenoxypyphenyl)acetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (19). 3-Phenoxypyphenylacetic acid (170 mg, 0.74 mmol), oxalyl chloride (76 μL, 0.88 mmol), and DMF (1 drop) were reacted in DCM (3 mL) according to method C. The resulting acid chloride and 7-aminocephalosporanic acid (0.37 mmol) were reacted in DCM (5 mL) prior to the addition of aniline (75 μL) according to method B. The aqueous layer was extracted with DCM (x3), and the organic layers were combined, dried over Na₂SO₄, and evaporated. The resulting solid was precipitated from hot DCM to afford the product as a white amorphous solid (22 mg, 12%). IR (solid): 3280, 3042, 1771, 1726, 1659, 1528, 1226 cm⁻¹. HRMS (ESI+): calcd for C₂₄H₂₃N₂O₇S (M + H)⁺ 483.1226, found 483.1233.

(6R,7R)-3-(Acetoxymethyl)-7-benzamido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (20). 7-Aminocephalosporanic acid (100 mg, 0.37 mmol) and benzoyl chloride (51 μL, 0.44 mmol) were reacted in sat. NaHCO₃ (aq) (10 mL) and DMF (1 drop) were reacted in DCM (3 mL) according to method A to afford the product as a white amorphous solid (75 mg, 55%). IR (solid): 3254, 1774, 1752, 1710, 1651, 1520, 1223 cm⁻¹. HRMS (ESI+): calcd for C₁₉H₂₁N₂O₆S (M + H)⁺ 483.1226, found 483.1233.

(6R,7R)-3-(Acetoxymethyl)-7-(4-methylbenzamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (21). 4-Methylbenzyl chloride (97 μL, 0.74 mmol) were reacted in sat. NaHCO₃ (aq) (10 mL) and acetone (5 mL) according to method A to afford the product as a white amorphous solid (39 mg, 26%). IR (solid): 3254, 1774, 1730, 1684, 1525, 1223 cm⁻¹. HRMS (ESI+): calcd for C₁₉H₂₁N₂O₆S (M + H)⁺ 483.1226, found 483.1233.
162.1, 129.7, 125.1, 113.6, 62.9, 59.7, 57.7, 55.4, 25.0, 20.6. HRMS (ESI\(^{+}\)): calcd for C\(_{16}\)H\(_{23}\)N\(_2\)O\(_6\)S (M + H\(^{+}\)\) 357.1277, found 357.1277.

\((6R,7R)-3-(Acetoxymethyl)-7-butyramido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (26)\). Butyl chloride (76 \(\mu\)L, 0.74 mmol) and 7-aminoocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 \(\mu\)L) according to method B. The aqueous layer was extracted with DCM (3), and the organic layers were combined, dried over Na\(_2\)SO\(_4\), and evaporated. The resulting solid was triturated with ice-cold DCM to afford the product as a white amorphous solid (15 mg, 12%). IR (solid): \(\nu\)max 3297, 2982, 1774, 1718, 1528, 1368, 1223 \(\text{cm}^{-1}\). HRMS (ESI\(^{+}\)): calcd for C\(_{22}\)H\(_{27}\)N\(_3\)O\(_5\)F (M + H\(^{+}\)\) 422.0658, found 422.0653.

\((6R,7R)-3-(Acetoxymethyl)-7-hexamido-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (27)\). Hexanoyl chloride (103 \(\mu\)L, 0.74 mmol) and 7-aminoocephalosporanic acid (100 mg, 0.37 mmol) were reacted in EtOAc (5 mL) prior to the addition of aniline (100 \(\mu\)L) according to method B. The resulting precipitate was collected by vacuum filtration and washed with ice-cold DCM to afford the product as a white amorphous solid (78 mg, 57%). IR (solid): \(\nu\)max 3297, 2982, 1774, 1718, 1528, 1368, 1223 \(\text{cm}^{-1}\). HRMS (ESI\(^{+}\)): calcd for C\(_{23}\)H\(_{28}\)N\(_3\)O\(_6\)S (M + H\(^{+}\)\) 437.1277, found 437.1290.

**tert-Butyl (6R,7R)-7-Acamidom-3-(acetoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (28)\). Compound 26 (75 mg, 0.24 mmol) was dissolved in DCM (2 mL), tert-butyl 2,2-trichloroacetimidate (170 \(\mu\)L, 0.96 mmol) was added, and the reaction was heated to 60 °C for 24 h. After cooling to room temperature, the reaction was diluted with MeOH and MeOH. Solvent was removed under reduced pressure and the resulting solid triturated with cold DCM. The solute was loaded directly onto a 10 g SNAP KPSI column and purified by column chromatography (0–10% MeOH in DCM) to afford the product as a creamy glassy solid (82 mg, 93%). IR (thin film): \(\nu\)max 3297, 2982, 1774, 1718, 1670, 1528, 1368, 1223 \(\text{cm}^{-1}\). HRMS (400 MHz, CDCl\(_3\)) \(m/z\) 639.1 (\(\text{M}^{+}\)), 584.1 (\(\text{M}^{+}\)), 530.1 (\(\text{M}^{+}\)), 476.1 (\(\text{M}^{+}\)), 422.1 (\(\text{M}^{+}\)), 367.1 (\(\text{M}^{+}\)), 313.1 (\(\text{M}^{+}\)), 259.1 (\(\text{M}^{+}\)), 205.1 (\(\text{M}^{+}\)). HRMS (ESI\(^{+}\)) for C\(_{22}\)H\(_{27}\)N\(_3\)O\(_5\)S (M + H\(^{+}\)\): 371.1277, found 371.1276.

**2,4-Dimethoxy-5-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (32)\). Ciprofloxacin 31 (500 mg, 1.51 mmol) was dissolved in 1 M NaOH (aq) (5 mL) and THF (10 mL) added, followed by the dropwise addition of Bu\(_{2}\)NOH.HCl (306 mg, 3.12 mmol) to the THF (10 mL) and stirred at room temperature for 16 h. Solvent was removed under reduced pressure and the resulting material dissolved in H\(_2\)O and neutralized with sat. NH\(_4\)Cl (aq). The precipitate was collected by vacuum filtration and washed with H\(_2\)O to afford the product as a white amorphous solid (302 mg, 77%). IR (solid): \(\nu\)max (KBr) 3497, 2937, 2971, 1771, 1718, 1654, 1539, 1223 \(\text{cm}^{-1}\). HRMS (ESI\(^{+}\)) for C\(_{22}\)H\(_{27}\)N\(_3\)O\(_6\)S (M + H\(^{+}\)\): 432.1935, found 432.1951.
Sodium 7-[(4-tert-Butyloxycarbonyl)piperazin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (33). Compound 32 (105 mg, 0.240 mmol) was suspended in MeOH (2.44 mL), 0.1 M NaOH (aq) (2.44 mL) was added, and the reaction mixture was stirred at 30 °C for 30 min. Solvent was removed under reduced pressure and resulting material suspended in H2O (5 μL) and EtOH (5 mL) and evaporated to dryness (x3). Then, the solid was suspended in DCM and evaporated to afford the product as a cream amorphous solid (111 mg, quant.). IR (solid): νmax 1617, 1478, 1242 cm⁻¹.

tert-Butyl (6R,7R)-7-Acetamido-3-{{(1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxyloxy)methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (34). Compound 29 (198 mg, 0.54 mmol) was dissolved in DCM (8 mL) and TMSI (117 μL, 0.82 mmol) added dropwise.83 The reaction mixture was stirred in the dark for 2 h at room temperature, then diluted with DCM and washed with 10% (wt/v) Na2SO3 (aq). The organic layer was dried over Na2SO4 and evaporated to give compound 30 as a yellow glassy solid. Compound 30 (120 mg, 0.27 mmol) and compound 33 (100 mg, 0.30 mmol) were suspended in anhydrous 1,4-dioxane (3.5 mL), and DME (11.5 mL), was added dropwise. The reaction mixture was stirred in the dark for 2 h before the solvent was removed under a stream of N2. The resulting material was dissolved in minimal DCM and loaded directly onto a 10 g SNAP Ultra cartridge and purified by column chromatography (0–6% MeOH in DCM) to afford the product as a pale yellow glassy solid (108 mg, 52%).

**ACKNOWLEDGMENTS**

We thank the Imperial Confidence in Concept scheme and the Wellcome Trust (Pathfinder Award 204337/Z/16/Z) for funding this work. A.M.E. also acknowledges support from the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC). J.S. acknowledges funding from the Engineering and Physical Sciences Research Council (Grant EP/M027546/1) and the Biotechnology and Biological Sciences Research Council-funded South West Biosciences Doctoral Training Partnership (Grant Reference BB/ J014400/1, studentship to C.L.T.). T.B.C. is a Sir Henry Dale Fellow jointly funded by the Wellcome Trust and Royal Society (Grant 107660/Z/15/Z). We thank Peter Haycock from the Imperial College Department of Chemistry NMR Facility and Lisa Haigh from the Imperial College Department of Chemistry Mass Spec Facility for their support with NMR and mass spectrometry data collection, respectively. We thank Prof. Matthew B. Avison, University of Bristol, for providing the pSU18 vectors used in this study. We also thank Ali Abdolrasouli, Charing Cross Hospital, for providing the clinical isolates used in this study.

**ABBREVIATIONS USED**

E. coli, Escherichia coli; FDA, Food and Drug Administration; GI, gastrointestinal; GU, genitourinary; kcat**, catalytic constant for the conversion of substrate to product; P. aeruginosa, Pseudomonas aeruginosa; TMSI, trimethylsilyl iodide.

**REFERENCES**


(83) Reaction was performed under strict anhydrous conditions. Increasing the equivalents of TMSI or extending the reaction time led to decomposition of the product and a reduction in recovered material.