HIV Drugs Inhibit Transfer of Plasmids Carrying Extended-Spectrum β-Lactamase and Carbapenemase Genes

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ABSTRACT

Antimicrobial-resistant (AMR) infections pose a serious risk to human and animal health. A major factor contributing to this global crisis is the sharing of resistance genes between different bacteria via plasmids. The WHO lists Enterobacteriaceae, such as Escherichia coli and Klebsiella pneumoniae, producing extended-spectrum β-lactamases (ESBL) and carbapenemases as “critical” priorities for new drug development. These resistance genes are most often shared via plasmid transfer. However, finding methods to prevent resistance gene sharing has been hampered by the lack of screening systems for medium-/high-throughput approaches. Here, we have used an ESBL-producing plasmid, pCT, and a carbapenemase-producing plasmid, pKpQIL, in two different Gram-negative bacteria, E. coli and K. pneumoniae. Using these critical resistance-pathogen combinations, we developed an assay using fluorescent proteins, flow cytometry, and confocal microscopy to assess plasmid transmission inhibition within bacterial populations in a medium-throughput manner. Three compounds with some reports of antiplasmid properties were tested; chlorpromazine reduced transmission of both plasmids and linoleic acid reduced transmission of pCT. We screened the Prestwick library of over 1,200 FDA-approved drugs/compounds. From this, we found two nucleoside analogue drugs used to treat HIV, abacavir and azidothymidine (AZT), which reduced plasmid transmission (AZT, e.g., at 0.25 g/ml reduced pCT transmission in E. coli by 83.3% and pKpQIL transmission in K. pneumoniae by 80.8% compared to untreated controls). Plasmid transmission was reduced by concentrations of the drugs which are below peak serum concentrations and are achievable in the gastrointestinal tract. These drugs could be used to decolonize humans, animals, or the environment from AMR plasmids.

IMPORTANCE

More and more bacterial infections are becoming resistant to antibiotics. This has made treatment of many infections very difficult. One of the reasons this is such a large problem is that bacteria are able to share their genetic material with other bacteria, and these shared genes often include resistance to a variety of antibiotics, including some of our drugs of last resort. We are addressing this problem by using a fluorescence-based system to search for drugs that will stop bacteria from sharing resistance genes. We uncovered a new role for two drugs used to treat HIV and show that they are able to prevent the sharing of two different types of resistance genes in two unique bacterial strains. This work lays the foundation for future work to reduce the prevalence of resistant infections.

KEYWORDS

antimicrobial resistance, E. coli, K. pneumoniae, ESBL, carbapenemases, KPC, CTX-M, carbapenemase

The threat of untreatable infections due to antimicrobial resistance (AMR) has been recognized by global agencies, including the World Health Organization (WHO), and national governments (1–3). A key factor contributing to AMR is mobile genetic
elements such as plasmids, which can carry multiple antimicrobial resistance genes (ARGs) and are commonly found within the order Enterobacteriales (which consists of one family, Enterobacteriaceae), including many difficult-to-treat species such as multidrug-resistant (MDR) Escherichia coli and Klebsiella pneumoniae. In particular, plasmids increasingly carry ARGs coding for proteins such as extended-spectrum β-lactamases (ESBLs; e.g., CTX-M), carbapenemases (e.g., KPC), and colistin resistance proteins (e.g., MCR-1) (4–6). The WHO lists carbapenem-resistant and ESBL-producing Enterobacteriaceae as a critical priority for which new drugs are needed (7), and the CDC lists them as an urgent and serious threat, respectively (8). Therefore, finding new strategies to combat ESBL- and carbapenemase-producing Enterobacteriaceae is of the utmost importance.

These bacteria have proven particularly difficult to treat for a few key reasons; one is that the genes coding for ESBLs and carbapenemases are most often carried on plasmids, most of which are conjugative. These AMR plasmids and ARGs are found within, and shared between, bacteria colocalizing the same niche, such as intestinal tracts (9–11); environments, including wastewater and river sediments (12); agricultural soil (13–15); and hospital surfaces (16, 17). Furthermore, ARGs frequently located on plasmids have been acquired by people traveling to areas of the world with high levels of AMR (18–20). With the single acquisition of an MDR, ESBL- or carbapenemase-producing plasmid, Enterobacteriales can go from being easily treatable to extremely challenging. Therefore, AMR plasmids pose a serious risk to human and animal health.

One example of a highly successful AMR plasmid is the IncK plasmid pCT, which carries a group 9 blaCTX-M-14 ESBL gene (5). pCT-like elements have been found in commensal and extraintestinal pathogenic E. coli isolates from humans and animals (21, 22). The CTX-M family is the largest group of ESBLs (23). blaCTX-M-14 is the most commonly found ESBL gene in parts of Asia and Spain (23). IncK plasmids in particular have contributed to the spread of blaCTX-M-14 in Spain (24) and the United Kingdom (25). The high rate of pCT conjugation and high degree of stability have contributed to the spread of pCT-like plasmids among diverse E. coli strains (21, 26, 27). E. coli from the ST131 clonal group is globally the most predominant cause of extraintestinal E. coli infections, such as urinary tract infections (UTIs), and has been associated with the success of some blaCTX-M genes such as blaCTX-M-15 (23, 28, 29).

Likewise, the carbapenemase-producing plasmid pKpQIL and its variants have been isolated around the world in a variety of species but are predominantly found in K. pneumoniae (30–33). The prevalence and spread of pKpQIL in the United Kingdom have been well characterized (6, 34–38). pKpQIL is a 114-kb, IncFIK2 plasmid carrying the blaKPC carbapenemase gene, the blaTEM β-lactamase gene, and heavy metal resistance and is self-transmissible (30, 39, 40). pKpQIL is well adapted to the K. pneumoniae host and is stably maintained in K. pneumoniae populations (31, 38).

One approach to tackling ESBL- and carbapenemase-producing Enterobacteriales is to reduce the prevalence of AMR plasmids, by decolonization of people, animals, and/or the environment. Antiplasmid compounds can act by reducing plasmid stability, resulting in eradication of plasmids from a population (termed plasmid curing), and/or preventing transmission of a plasmid to a new host (41, 42). Except for a few such as chlorpromazine, ascorbic acid, and linoleic acid, most compounds identified since the 1960s with reported antiplasmid activity are toxic to humans (42). Chlorpromazine belongs to the phenothiazine class of antipsychotic drugs (43, 44). However, the literature regarding the plasmid curing ability of chlorpromazine is controversial, and there are inconsistent reports of activity. Ascorbic acid (vitamin C) cured Gram-positive bacteria (Staphylococcus aureus and Pediococcus acidilactici) of some drug resistance plasmids (45–47). However, the impact upon clinically relevant Gram-negative bacterium-plasmid host combinations is unclear. The unsaturated fatty acid linoleic acid inhibited conjugation of some plasmids in E. coli (48) by inhibiting the activity of the TwrD ATPase (VirB11 homologue), which is involved in the conjugative machinery required for transmission (49). However, none of these compounds have been tested on pCT or pKpQIL.
In order to discover safe and efficacious ways to remove and/or prevent the spread of ESBL- and carbapenemase-producing plasmids from Enterobacteriales, we have developed a method to monitor transmission of pCT and pKpQIL in real time using fluorescent protein genes on both the plasmids and host strains E. coli ST131 (50–52) and K. pneumoniae Ecl8 (38, 53, 54). Plasmid dynamics within the bacterial population were monitored using flow cytometry and microscopy. We demonstrate that chlorpromazine reduced pCT and pKpQIL transmission, while linoleic acid inhibited only pCT, and ascorbic acid had little impact. This assay was then used in a medium-throughput screen (MTS) of the Prestwick FDA-approved library. We identified a novel role as antiplasmid compounds for two FDA-approved anti-HIV drugs, abacavir and azidothymidine (AZT; also called zidovudine), which reduced transmission of both the ESBL- and carbapenemase-producing plasmids in E. coli and K. pneumoniae. We also demonstrated the activity of these novel antiplasmid compounds at clinically achievable concentrations and at which they do not impact bacterial growth, and so the likelihood of selective pressure for the emergence of transmission-inhibitor resistance is minimal.

RESULTS
Development of assay to measure plasmid transmission in a bacterial population. In order to rapidly measure the dynamics of pCT and pKpQIL transfer to host strains, fluorescence-based reporters were constructed in E. coli ST131 EC958 (clade C) and K. pneumoniae Ecl8, respectively. Prior to insertion of pCT into ST131 EC958, the resident plasmid (pEC958) (55) was removed using an incompatibility-based system (for additional detail, see Text S1 and Fig. S1a in the supplemental material).

The gfp gene was inserted into pCTCTX-M-14, giving pCTgfp, which was transferred by conjugation into ST131c, forming ST131c pCTgfp. In order to monitor pKpQIL in K. pneumoniae, the gfp gene was inserted into the blaKPC locus in pKpQIL, thus disrupting the blaKPC gene and resulting in pKpQILgfp. Conjugation was used to insert pKpQILgfp into K. pneumoniae strain Ecl8, forming Ecl8 pKpQILgfp. These strains formed the plasmid donors. The recipient strains were constructed by inserting mcherry into the putPA intergenic region, as per reference 56, in the chromosome of ST131c, thus forming ST131c mcherry, and of Ecl8, forming Ecl8 pKpQILgfp. Strains were confirmed by PCR and DNA sequencing (see Text S1 and Tables S4 to S6), and flow cytometry was used to confirm expression of fluorescent proteins (Fig. 1a). Plasmid transmission was measured by flow cytometry, which quantified the number of green fluorescent protein (GFP)-positive bacteria (donors), mCherry-positive bacteria (recipients), and GFP-positive/mCherry-positive bacteria (transconjugants) (Fig. 1b).

Since fluorescent proteins were used as an indicator of plasmid transmission, confocal microscopy was used to monitor plasmid transmission. Images of E. coli/pCTgfp donor and E. coli/mcherry recipient strains were collected at time zero, prior to transmission (Fig. 2a, top panel), and at 120 min, which was sufficient time for transmission events to occur and with transconjungant bacteria clearly visible (Fig. 2a, bottom panel). A time course was performed with the same strains by taking images every 10 min up to 120 min. During this time lapse, donor and recipient bacteria coming into close contact were captured at 60 and 90 min, as well as a transconjungant bacterium expressing both green and red fluorescent proteins at 120 min (Fig. 2b). Over 120 min, the number of colocalization events (defined as GFP+/mCherry+ pixels) was calculated and compared. An increase in the number of colocalization events was visible over time (from 0% to 0.13% of events, Fig. S1b). This indicates that plasmid transmission occurs rapidly and can be monitored by the fluorescent system.

Plasmid transmission is impacted by the ratio of donor and recipient bacteria. The ratio of donor to recipient cells is known to impact the transfer efficiency of plasmids (57–59). Therefore, we used flow cytometry to measure the number of transconjungant/transconjungant daughter cells in a population after coincubation of donors and recipients at a range of donor-to-recipient ratios and time points. All bacteria were adjusted to an optical density of 0.5 at 600 nm (OD600) prior to
conjugation experiments. For pCTgf<em>p</em> in <em>E. coli</em>, the highest number of transconjugants was observed after 24 h between ratios of 1:1 and 6:1 (Fig. 3a), with the number of transconjugants between 25 and 30% of cells. Based on these data, the optimal transmission ratio of 3 donor to 1 recipient was chosen for further experiments. To determine the optimal time point, transmission was measured every hour for 12 h and at 24 h using the 3:1 donor/recipient ratio. The level of transmission increased over time, with maximal numbers of transconjugants obtained between 11 and 24 h of coincubation (Fig. 3b).

For pKpQILgf<em>p</em> transmission in <em>K. pneumoniae</em>, after 6 h the number of transconjugants peaked between the ratios of 1:8 and 1:2 (Fig. 3c). Ratios between 1:1 and 8:1 resulted in a sequential reduction in transconjugant populations (Fig. 3c). The ratio of 1:2 was selected for further experiments. The number of transconjugants produced was monitored at three time points: 4, 6, and 24 h. There was little change in the number of transconjugants between 4 and 24 h (Fig. 3d). Six hours was chosen for further experiments because at 24 h evidence of biofilm formation was observed, which is known to impact plasmid transmission (60–62).

**Effect of known plasmid-transmission inhibitors upon transmission of pCT and pKpQIL into <em>E. coli</em> and <em>K. pneumoniae</em>, respectively.** (i) Chlorpromazine. Chlorpromazine reduced transmission of both plasmids in both strains. For pCTgf<em>p</em> transmission in <em>E. coli</em>, after 24-h exposure concentrations of chlorpromazine of ≥20 μg/ml significantly reduced transmission (<em>P</em> < 0.001, Fig. 4a). The MIC of chlorpromazine was 128 μg/ml for all <em>E. coli</em> strains (Table 1). While the concentrations that inhibited transmission were below the MIC, we wanted to determine if chlorpromazine inhibited bacterial growth (as this may impact the composition of the population). Therefore, growth rate was determined in the presence of 30 μg/ml chlorpromazine. Some degree of growth inhibition was indicated by an increase in the generation time from 66 ± 8 min to 87 ± 10 min for ST131c, 75 ± 16 min to 96 ± 18 min for ST131 <em>mcherry</em>, and...
77 ± 20 min to 93 ± 14 min for ST131 c pCTf<sup>gfp</sup>. Additional growth kinetic experiments revealed that a chlorpromazine concentration of 20 µg/ml had no impact upon growth rate of <i>E. coli</i> ST131 EC958 derivatives (Fig. S2a). Furthermore, 20 µg/ml chlorpromazine still reduced pCT<sup>gfp</sup> transmission (Fig. 4a).

Concentrations of ≥50 µg/ml chlorpromazine resulted in a significant reduction in <i>K. pneumoniae</i> pKpQIL<sup>gfp</sup> transconjugants (Fig. 4b). The MIC of chlorpromazine for these strains was 256 µg/ml (Table 1). As with <i>E. coli</i>, 20 µg/ml chlorpromazine had no effect upon bacterial growth (Fig. S2b); however, this concentration did not reduce pKpQIL transconjugants. The concentration range that we tested goes above the peak serum concentrations of chlorpromazine (0.1 µg/ml) (63). We aimed to test concentrations of compounds that were within the therapeutic window. However, for some compounds such as chlorpromazine, only at higher concentrations were effects seen on bacterial plasmid transmission.

In order to validate the flow cytometry data, classical conjugation experiments with <i>E. coli</i> were performed. To enable these experiments, a rifampin-resistant recipient strain of ST131c was generated; PCR and sequencing indicated a mutation resulting in the substitution H537Y in the <i>rpoB</i> product. The MIC of rifampin for the wild type was 8 µg/ml and for the mutant was 1,024 µg/ml. The impact of chlorpromazine upon pCT<sup>gfp</sup> transmission was tested in liquid mating experiments, and the data obtained confirmed the results obtained by flow cytometry. In 20 µg/ml chlorpromazine, there was an 82% reduction in transconjugants compared to LB alone (Table 2). While the growth rate of the bacterial strains in 20 µg/ml chlorpromazine was unaffected, a 2.8-fold reduction in the viable counts of total bacteria after the classical conjugation assay was seen. This supported the hypothesis that chlorpromazine inhibits bacterial growth.

(ii) Linoleic acid. The unsaturated fatty acid linoleic acid was tested for inhibition of plasmid transmission. For <i>E. coli</i>, between 3 and 6 mM linoleic acid effectively reduced transmission of pCTf<sup>gfp</sup> at 24 h (3 and 5 mM, <i>P</i> ≤ 0.05; 4 and 6 mM, <i>P</i> < 0.001) (Fig. 4c). The MIC of linoleic acid was ≥512 mM for all strains (Table 1); 6 mM linoleic acid had no effect upon growth of the ST131c strains (Fig. S2a). Estimates for the normal serum levels of linoleic acid in healthy individuals vary between studies but are in the range of 0.2 to 5.0 mM (64, 65). The classical conjugation assays confirmed a 99% reduction...
in transconjugants compared to LB alone (Table 2), without impacting the viable counts of either ST131c pCTgfp or ST131c mcherry.

For pKpQILgfp transconjugants in *K. pneumoniae*, at 0.5 and 1 mM linoleic acid increased the number of transconjugants at 6 h ($P = 0.047$ and $<0.001$, respectively; Fig. 4d). No reduction in transconjugants was observed at any concentration tested. These concentrations are within the range which reduces transmission of other plasmids (48), including pCT (Fig. 4c), and are well below the MIC of linoleic acid (>512 mM, Table 1); 6 mM linoleic acid had no impact upon bacterial generation time (Fig. S2b).

(iii) Ascorbic acid. Ascorbic acid had no detectable impact on pCTgfp transmission in *E. coli* ST131c at any concentration when tested by flow cytometry up to the 24-h endpoint (Fig. 4e). However, ascorbic acid reduced the number of transconjugants when tested in a classical conjugation experiment (50% reduction [Table 2]). In order to identify antiplasmid activity of ascorbic acid, a concentration range at and above the upper peak serum concentration was used. The maximum concentration tested (3.5 mg/ml) was just below the MIC of ascorbic acid, 4 mg/ml (Table 1), and this concentration inhibited growth (increased the generation time of all strains [Fig. S2a]) and is above the peak serum concentration after intravenous (i.v.) treatment (2.4 mg/ml) (66). Ascorbic acid had no impact upon transmission of pKpQIL in *K. pneumoniae* (Fig. 4f), even at the high and growth-inhibiting concentration of 3.5 mg/ml; the MIC of ascorbic acid is 4 to 8 mg/ml (Table 1; Fig. S2b).

An ESBL-free ST131 clinical isolate shows a similar pCT transmission profile as EC958. To ensure that data obtained with *E. coli* ST131 EC958, which had been cured of its original plasmid, were not atypical, the effect of the antiplasmid compounds upon pCT transmission in a human clinical isolate of *E. coli* ST131 B104 with no identified plasmids (52) was measured. Both EC958 and B104 belong to ST131 clade C (52). For
B104, a donor-to-recipient ratio of 3:1 resulted in the most consistent and highest average proportion of transconjugants (Fig. 5a). These data are similar to those for \(p\)CT\(gfp\) transmission in ST131c. This ratio was therefore used for further experiments. Linoleic acid (6 mM) and chlorpromazine (20 \(\mu\)g/ml) both significantly reduced the number of transmission events at 24 h (\(P < 0.001\), Fig. 5b). The compounds had no effect on the growth of the strains (Fig. S2c). Overall, the data obtained were similar to those obtained for ST131c. Interestingly, ascorbic acid, which had no impact on \(p\)CT\(gfp\) transmission in ST131c, reduced transmission in B104 by 19% compared to LB alone (\(P < 0.05\), Fig. 5b).

Medium-throughput screening of the Prestwick library identified two FDA approved drugs with antiplasmid activity in both \(E. coli\) and \(K. pneumoniae\).
Ascorbic acid (5.62 mM), chlorpromazine (20 μg/ml), abacavir (8 μg/ml), or AZT (0.008 μg/ml) was added to ST131c in the presence of an antibiotic or compound. The resistance to these antibiotics is encoded on the pEC958 plasmid. The resistance to this antibiotic is encoded on the ST131 EC958 chromosome. AZT-resistant mutant ST131c pCTgfp aZT-resistant mutant ST131c pCTgfp

(i) Abacavir. Abacavir did not inhibit growth of any bacterial strain (MIC of >512 μg/ml, Table 1), and 32 μg/ml of abacavir had no effect on the growth of any strain (Fig. S2a and b). To determine the optimal concentration for transmission

TABLE 2 Conjugation frequencies of pCTgfp from ST131 EC958 pCTgfp into ST131c in the presence of ascorbic acid (3.5 mg/ml), linoleic acid (6 mM), chlorpromazine (20 μg/ml), abacavir (8 μg/ml), or AZT (0.008 μg/ml) a

| Compound          | Conjugation frequency in compound (± SD) | Corresponding conjugation frequency in LB control (± SD) | % conjugation compared to LB control |
|-------------------|-----------------------------------------|--------------------------------------------------------|-------------------------------------|
| Ascorbic acid     | 5.62 x 10^{-4} ± 9.47 x 10^{-5}         | 1.15 x 10^{-7} ± 2.24 x 10^{-7}                       | 49                                  |
| Linoleic acid     | 8.48 x 10^{-10} ± 3.28 x 10^{-10}       | 1.15 x 10^{-7} ± 2.24 x 10^{-7}                       | 0.74                                |
| Chlorpromazine    | 4.24 x 10^{-9} ± 2.53 x 10^{-9}         | 2.32 x 10^{-7} ± 1.74 x 10^{-7}                       | 18                                  |
| Abacavir          | 6.22 x 10^{-7} ± 2.90 x 10^{-7}         | 4.61 x 10^{-7} ± 2.55 x 10^{-7}                       | 135                                 |
| AZT               | 2.34 x 10^{-7} ± 6.16 x 10^{-9}         | 3.25 x 10^{-7} ± 1.39 x 10^{-7}                       | 72                                  |

aData are the averages from three independent experiments, carried out with a minimum of three biological replicates each. Corresponding conjugation frequency in LB control represents frequency for the particular set of three independent experiments.
inhibition, dose-response curves with abacavir were performed using flow cytometry. These demonstrated significant reduction of pCT<sub>gfp</sub> transmission in ST131c at 8 μg/ml abacavir (85.4% of the LB controls, \(P < 0.01\)) (Fig. 6a). Interestingly, 16 μg/ml had no impact on pCT<sub>gfp</sub> transmission (Fig. 6a). For <i>E. coli</i> ST131 B104, 16 μg/ml abacavir resulted in a significant reduction of pCT<sub>gfp</sub> transmission (85% of LB controls, \(P = 0.0036\)) (Fig. S3). Concentrations of >32 μg/ml abacavir affected recipient strain growth (data not shown). Classical conjugation assays showed that 8 μg/ml abacavir increased the number of transconjugants by 135% (Table 2). Abacavir is a well-characterized drug, and concentrations reached in the serum of patients being treated for HIV are typically 3 μg/ml (Table S1).

(ii) AZT. AZT was also identified in our MTS experiments. Using flow cytometry, AZT caused a substantial, highly significant, and dose-dependent reduction in pCT<sub>gfp</sub> transmission in <i>E. coli</i> (Fig. 6c); even the lowest concentration of AZT tested (0.008 μg/ml) resulted in a 52.4% reduction compared to LB alone (\(P = 0.0067\)); 0.25 μg/ml AZT reduced transconjugants by 83.3% ± 4.9% of LB controls (\(P < 0.0001\)). An AZT concentration of 0.008 μg/ml is between 1/512 and 1/256 the MIC of AZT for both strains (Table 1). As other studies have examined the antimicrobial activity of AZT (67–71), we also examined the impact of sub-MICs of AZT on bacterial growth. With increasing AZT concentrations, there was an increase in the generation time; at 0.008 μg/ml there was no effect on bacterial growth (Fig. S2d and e). This is well below peak serum concentrations, which range between 0.05 and 1.46 μg/ml (Table S1) (72). Together, this shows a concentration of AZT which reduces transmission of pCT by ~50% and has no effect on bacterial growth. Supporting this, the classical conjugation experiments revealed that at 0.008 μg/ml, the conjugation frequency with AZT was 28% lower than the conjugation frequency in LB alone (Table 2).

![FIG 5](image-url)  
**FIG 5** pCT<sub>gfp</sub> transmission dynamics in ST131 strain B104. (a) The impact of donor/recipient ratio on pCT<sub>gfp</sub> transmission. Each data point is the mean from four biological replicates; each dataset comprises four independent experiments. (b) The impact of 6 mM linoleic acid, 3.5 mg/ml ascorbic acid, or 20 μg/ml chlorpromazine on pCT<sub>gfp</sub> transmission in B104 populations. Data show the mean ± standard deviation from four independent experiments, each with four biological replicates. *, \(P < 0.05\); **, \(P < 0.001\), calculated using Student’s t test.
There was a large reduction in the number of pKpQIL K. pneumoniae transconjugants formed in the presence of 0.008 to 0.25 μg/ml AZT (Fig. 6d); 0.25 μg/ml AZT reduced transconjugants by 80.8% ± 3.4% of LB controls (P < 0.0001). The MICs of AZT for the K. pneumoniae strains were 4 and 2 μg/ml for the Ecl8 parental strain and Ecl8 pKpQILgfp, respectively, and the MIC was 16 μg/ml for Ecl8 mcherry. We consistently saw variation in the MIC between biological replicates within each experiment, with sporadic mutants appearing in wells with higher concentrations of AZT. In order to quantify this observation, we determined the mutation frequency of E. coli and K. pneumoniae strains to AZT (Table S2), which ranged from $8.65 \times 10^{-7}$ to $1.45 \times 10^{-6}$. This is in line with the observations of others (67, 68, 71, 73). Ecl8 mcherry grew more...
quickly at 0.016 and 0.032 µg/ml AZT, while above 0.12 µg/ml AZT inhibited growth (Fig. S2 d and e). Ecl8/pKpQILgfp grew more slowly at ≥0.06 µg/ml AZT (Fig. S2 d and e). At the lowest concentrations tested (0.008 to 0.032 µg/ml), there was no effect on growth of donors or recipients and these concentrations significantly reduced pKpQIL transmission (Fig. 6 d).

AZT is a prodrug which is activated within the cell by phosphorylation events mediated by thymidine kinase to produce AZT-triphosphate, the active component in DNA chain termination (74). Single point mutations in thymidine kinase can result in AZT resistance (68, 73). Therefore, to determine if AZT-triphosphate was the active antiplasmid compound, we selected AZT-resistant E. coli mutants after exposure to 16 µg/ml AZT for 24 h. The mutation frequency to AZT (Table S2) was typical of that associated with a mutation in a single gene. The MIC of AZT for the mutants was >32 µg/ml. Sequencing of the thymidine kinase gene revealed a 10-bp deletion. The AZT-resistant mutants were then used in AZT transmission assays. AZT had no effect on plasmid transmission in the AZT-resistant mutants (Fig. 6 e). This indicates that the active antiplasmid component is AZT-triphosphate.

**DISCUSSION**

In this study, the dynamics of two globally disseminated, clinically relevant plasmids, producing either an ESBL or carbapenemase, were monitored for transmission in near real time into E. coli and K. pneumoniae populations, respectively. With fluorescently labeled plasmids and recipient bacteria, we monitored plasmid transmission among populations using flow cytometry.

We showed that chlorpromazine and linoleic acid function as inhibitors of an IncK CTX-M-producing plasmid transfer in E. coli and that chlorpromazine also functions as an antiplasmid compound for the IncFII KPC-producing plasmid pKpQIL in K. pneumoniae. Ascorbic acid had little/no effect on plasmid transmission, despite having a strong impact on bacterial growth. This shows that compounds that decrease growth do not necessarily also reduce plasmid transmission. While a variety of factors, including growth rate and low-level antibiotic selection, can impact the frequency of plasmid transfer (75, 76), we did not see such impact with ascorbic acid. Much of the work demonstrating the antiplasmid activity of ascorbic acid has been done with Gram-positive bacteria (45–47) and not Gram-negative bacteria as used here.

Chlorpromazine is a bioactive molecule which has many effects on the bacterial cell (63). These include intercalation into DNA molecules and possible breaks in single-stranded DNA (ssDNA) (77, 78). This intercalation could prevent the replication of the plasmid, resulting in plasmid loss from a population of bacteria. Chlorpromazine integrates into the lipid bilayer, impacting membrane permeability, fluidity, and some membrane-associated proteins (79–81). For example, chlorpromazine disrupted the activity of membrane-associated ATPases (82, 83). One can speculate that chlorpromazine may impact either the plasmid DNA itself, or the activity of the ATPases involved in the type IV secretion system used for conjugation, and thus inhibit plasmid transmission.

Here, we show that linoleic acid effectively inhibited conjugation of the IncK plasmid pCT at 24 h but had no impact on the IncFII plasmid pKpQIL in K. pneumoniae. Linoleic acid has previously been shown to inhibit conjugation of some plasmids, including IncF and IncW (48). Linoleic acid prevents conjugation by inhibiting the activity of the R388 plasmid TrwD ATPase, which is a type IV secretion traffic ATPase, and a homologue of Agrobacterium tumefaciens VirB11 (49, 84). The pCT plasmid encodes an ATPase in trbB (5), which in the plasmid RP4 encodes a homologue of VirB11 (85). Therefore, we hypothesize that the pCT TrbB is the target of linoleic acid-mediated transmission inhibition.

There was good correlation between the pCT flow cytometry data and the classical conjugation data. Both assays show that linoleic acid had the strongest antiplasmid activity, followed by chlorpromazine, AZT, and then abacavir. Interestingly, ascorbic acid reduced the conjugation frequency in classical conjugation
experiments, but this was not detected in the ST131c flow cytometry experiments. It may be that the low level of activity of ascorbic acid is near the limit of detection of the flow cytometry assay. We hypothesize that any discrepancy between the conjugation assay and the flow cytometry assay may also relate to the large variation between conjugation assay experiments. Overall, the flow cytometry assay is optimal for medium-high-throughput experiments to identify compounds that inhibit plasmid transmission.

The flow cytometry assay was used to screen in medium throughput (hundreds of compounds/week) for compounds/drugs that inhibited transmission of pCT and pK-pQIL within two species of bacteria. The two most promising hits from the Prestwick library were abacavir and AZT. While the impact of abacavir was modest, AZT had potent activity. These compounds have very different chemical structures from conjugation inhibitors such as linoleic acid and chlorpromazine. Abacavir and AZT are nucleoside analogue prodrugs that inhibit reverse transcriptase activity and are used clinically to treat HIV infection (see Table S1 in the supplemental material) (74, 86–88). AZT is converted into the active metabolite AZT-5'-triphosphate by thymidine kinases (67, 68), which is incorporated into DNA in place of thymidine, but since AZT lacks the 3'-hydroxy group, it results in chain termination (74). In the 1980s, AZT (2 μg/ml) was shown to inhibit the growth of E. coli and K. pneumoniae; however, AZT-resistant mutants with mutations in thymidine kinase were easily isolated in vitro (67); we made a similar observation in this study. Furthermore, AZT-resistant E. coli and Salmonella were recovered from patients receiving AZT treatment for HIV infection (89, 90). Recently, and in light of growing AMR, the antimicrobial properties of AZT have been explored (68–71, 73).

We hypothesize that the mechanism of antiplasmid activity of abacavir and AZT is that these compounds interfere with plasmid replication, including DNA chain termination, thus reducing plasmid transfer and/or plasmid presence in the population. This would explain our conjugation frequency data, which showed a smaller reduction in conjugation frequency in the presence of AZT than suggested by flow cytometry at the same concentration. Thus, when AZT reduces plasmid prevalence within the bacterial population, a substantial portion of cells may have lost the plasmid with the selectable marker (aph) used to distinguish donor/recipient/transconjugant bacteria; thus, in conjugation experiments such cells would skew the calculations used to determine conjugation frequency.

Two independent screens of FDA-approved compound libraries identified AZT as having activity against drug-resistant Gram-negative bacterial isolates (69, 70) and obtained MICs of AZT similar to those found in our study. The concentrations required to kill bacteria are within the peak serum concentrations for the drug (Table S1), and the use of already-approved drugs as new antibacterial compounds is attractive, as much of the pharmacokinetics (PK) and safety profiles are well established (74). In a study on human intestinal flora, 20 μM AZT affected growth of 10 bacteria including some strains of Bacteroides, Bifidobacterium, Clostridium, Odoribacter, Roseburia, and E. coli (91). Maier et al. (91) also estimated the intestinal concentrations of abacavir and AZT to be around 150 μM and 374 μM, respectively. These concentrations are much higher than the plasma concentrations of both drugs (Table S1) and, importantly, much higher than the concentrations which have antiplasmid properties. The concentration at which we saw antiplasmid activity (8 μg/ml abacavir corresponds to 27.9 μM; 0.008 μg/ml AZT corresponds to 0.029 μM) is within this range. Drug accumulation in the gut is ideal for an antiplasmid compound, as the intestinal flora is considered to be a hot spot for horizontal gene transfer, and dosage could be minimized, thus reducing potential systemic adverse effects. AZT treatment for HIV infection can result in AZT-resistant bacteria (89, 90). However, since the concentration which inhibits AMR plasmids is low (~1/512 the MIC), the use of low doses as antiplasmid strategies would minimize the selection of resistant mutants. Furthermore, it is possible that although resistant bacteria could be selected at higher concentrations, such bacteria may no longer harbor plasmids carrying ARGs. To explore this
hypothesis, clinical isolates of AZT-resistant bacteria should be examined for the presence of plasmids.

AMR plasmids are frequently found in the normal bacterial flora, in particular in the gastrointestinal (GI) tract (10, 11, 18–20, 41, 42). While it is clear that AMR plasmids are found and can persist within the GI tract, details of the intricacies of the AMR plasmid-microbiome relationships are still unclear. The impact of antiplasmid compounds on the AMR plasmid-microbiome relationship, including the impact of plasmid copy number and evolution, remains to be elucidated. However, we anticipate that improved models of AMR plasmid dynamics in the microbiome will allow the opportunity to address the impact of these factors on antiplasmid compound efficacy. In future, the testing of compounds such as abacavir and AZT in multispecies experiments will provide important insight into potential pathogen-microbiome interactions.

Before antiplasmid compounds can be used to reduce AMR prevalence in bacterial populations, additional factors should be considered. These include the potential differential activities of compounds on different plasmids and hosts. As shown here, compound activity may not be ubiquitous against all plasmids/bacteria. Furthermore, clinical strains can contain multiple plasmids; the impact of compounds in these settings should be examined. In complex, multispecies environments, plasmid-containing strains may also form the minority within the population; therefore, impact of compounds on minority populations and plasmid-free cells should be considered.

Additional chemical modifications and/or homologues may improve the antiplasmid activity and/or reduce antibacterial properties of abacavir and AZT, thus improving activity and alleviating any potential for selection pressure for AZT resistance. The use of nontoxic compounds to limit the spread of AMR plasmids (as opposed to killing the bacteria and thus reducing selective pressure) in key areas where transmissible ARGs are prevalent could be an effective means of reducing critical AMR in bacteria.

Identification of antiplasmid compounds has been slow, in large part due to dependence upon laborious plating experiments, which are not amenable to medium-/high-throughput screening. Apart from one screening system for conjugation inhibitors (48, 92, 93), most studies of antiplasmid compounds have used classical conjugation experiments. We developed and applied a fluorescence-based assay to monitor the movement of globally prevalent plasmids associated with human infections in two different Gram-negative species with plasmids carrying ESBL or carbapenemase genes. Furthermore, we used this assay in a medium-throughput screen and identified novel antiplasmid compounds. We uncovered the antiplasmid properties of two currently licensed and widely used anti-HIV drugs, abacavir and AZT. In light of the challenges associated with developing new antibiotics, nucleoside analogue drugs could be used to reduce the prevalence of drug-resistant Gram-negative bacteria via decolonization of vulnerable patients so that if they succumb to an infection a currently licensed drug will be effective.

**MATERIALS AND METHODS**

**Bacterial strains, growth, and measurement of drug susceptibility.** The ST131 strain EC958 was isolated from a patient with a community-acquired UTI in Northwest England and has been used as a representative of this clonal group (50, 51). It is a multidrug-resistant (MDR) strain, carrying a pEK499-like 136-kb plasmid, pEC958 (50, 51). pEC958 is an IncF plasmid with two replicons, RepFII and RepFIA, and 12 resistance genes, including CTX-M-15 (55). The pKpQIL plasmid was used as previously described (termed pKpQIL-UK in publications [38, 94]). Repeated attempts to establish the fluorescent system in K. pneumoniae from the ST258 lineage were unsuccessful. Therefore, we used K. pneumoniae Ecl8, which has been used previously (38, 53, 54) by others and ourselves for successful cloning applications. Details regarding sequencing, culture conditions, growth kinetics assays, MIC assays, and pEC958 curing can be found in Text S1 and the list of plasmids, strains, and primers can be found in Table S3, both in the supplemental material.

**Construction of gfp reporters.** pCTCTX-M-14 was previously modified by insertion of gfp-aph under the control of the acpP promoter into blaCTX-M-14 and stored in E. coli DH5α (95). Filter matings (per reference 38) were used to move pCTgfp from DH5α into ST131c, the variant of ST131 EC958 cured using
an incompatibility system (courtesy of Christopher M. Thomas), and transconjugants were selected on 256 μg/ml nalidixic acid and 75 μg/ml kanamycin. Plasmid presence was confirmed by PCR, and host strain identity was confirmed by testing ciprofloxacin resistance, confirming previously reported MIC values (55), and whole-genome sequencing (WGS; details available in Text S1). Expression of gfp was confirmed by flow cytometry, as described below.

For pKpQILgfp construction, pUA66 was used as the template, and PCR was used to amplify gfp-mut2 under the control of the acpP promoter with aph located downstream of gfp-mut2. E. coli SW105 containing temperature-sensitive recombinase and pKpQIL was made electrocompetent, and gfp-mut2 PCR product was transformed into the cells. Successful insertions were selected on kanamycin (50 μg/ml) and confirmed by PCR and sequencing. The constructed plasmid pKpQILgfp was transformed into E. coli DH10B for long-term storage. K. pneumoniae Ecl8 was made electrocompetent, and pKpQILgfp was transformed into cells, selected for using 50 μg/ml kanamycin, and confirmed by PCR and sequencing. Expression of GFP was confirmed by flow cytometry.

Construction of mCherry-expressing recipient strains. To prepare the mcherry fragment, the acpP promoter was amplified from pUA66 (L1019), using primers with HindIII and BamHI sites; this was then digested and inserted into mcherry encoding pET-17b. This resulted in pET-17b pacpP-mcherry. The aph cassette was amplified from pKD4, with primers inserting XhoI sites up- and downstream of the gene. Restriction digestion and ligation were carried out to insert aph downstream of mcherry. PCR was used to confirm the direction of the aph cassette, and the final construct (pET-17b pacpP-mcherry-aph) was confirmed by PCR and sequencing. PCR was used to amplify the pacpP-mcherry-aph region, with primers containing a 45-bp overlap corresponding to the putPA intergenic region in E. coli or K. pneumoniae. putPA was chosen because this locus has been previously shown to minimize impact and allow expression of fluorescent proteins (56).

The pSIM18 plasmid, expressing recombinase machinery under a temperature-sensitive promoter (96), was transformed into electrocompetent E. coli or K. pneumoniae. pSIM18-containing colonies were selected using 150 μg/ml hygromycin at 30°C. Expression of the recombinase machinery was induced, and pSIM18 cells were made electrocompetent; the pacpP-mcherry-aph PCR product was transformed into these cells. After recovery at 37°C, cells were subcultured on plates containing 50 μg/ml kanamycin. Successful insertion events were identified by PCR amplification of the inserted region, and loss of hygromycin resistance (pSIM18) was tested. Expression of mcherry was confirmed by flow cytometry, as described below.

Construction of fluorescent reporters in E. coli ST131 B104. A naturally plasmid-free E. coli ST131 clinical isolate, B104 (52), was modified for use in the pCT transmission assay. First, to construct the B104 donor strain, a rifampin-resistant B104 strain was generated by overnight growth on LB agar containing 100 μg/ml rifampin. Candidate clones were selected, and growth kinetics were determined to ensure that mutation did not impact growth rate. Sequencing of the rpoB gene was performed to identify the mutations resulting in rifampin resistance. Then, conjugation was used to move pCTgfp from ST131c pCTgfp into B104pCTgfp. PCR was used to confirm the presence of pCTgfp, and flow cytometry was used to check fluorescence. To construct the B104 recipient strain, the recombineering plasmid pST5S (97) was first transformed into B104 by electroporation, as described above. PCR was used to amplify the pacpP-mcherry-aph region from pET-17b pacpP-mcherry-aph, with primers containing a 45-bp overlap corresponding to the putPA intergenic region in B104. This product was transformed into electrocompetent B104 pSLTS, similar to what is described above and according to reference 97. Successful recombination events were selected on 50 μg/ml kanamycin. PCR was used to confirm insertion of mcherry, and flow cytometry was used to check fluorescence.

Transmission assay. Overnight cultures of donor (E. coli with pCTgfp or K. pneumoniae with pKpQILgfp) and recipients (E. coli or K. pneumoniae with chromosomal mcherry) were washed in phosphate-buffered saline (PBS) (Sigma) and diluted to an optical density (600 nm) of 0.5. Bacteria were combined at a donor/recipient ratio of 3:1 for E. coli and 1:2 for K. pneumoniae, unless otherwise specified. This mixture (20 μl) was used to inoculate 180 μl of LB broth in a 96-well plate. Controls included LB broth alone, donor alone, and recipient alone. The plate was incubated at 37°C with gentle agitation (~80 rpm) for the specified amount of time. At each time point, 20 μl was removed and serially diluted to 1:1,000 in PBS filtered through 0.2-μm filters. Samples were run on the Attune NxT acoustic focusing flow cytometer with Autosampler (Thermo Scientific), equipped with a blue/yellow (excitation laser: blue, 488 nm; yellow, 561 nm) laser configuration; additional details can be found in Text S1. Flow cytometry has been previously used to monitor plasmids within populations (for examples, see references 98 and 99).

Ascorbic acid, chlorpromazine, linoleic acid, abacavir, and AZT were added to LB in 96-well plates, over a range of concentrations. Ascorbic acid stock solutions of 100 mg/ml were prepared in sterile distilled water, chlorpromazine stock solutions of 10,000 μg/ml were prepared in water, linoleic acid stock solutions of 100 mM were prepared in 30% dimethyl sulfoxide (DMSO), abacavir sulfate stock solutions of 10,000 μg/ml were prepared in water, and AZT stock solutions of 1,000 μg/ml were prepared in methanol. All transmission assays were completed with a minimum of three independent experiments, with between three and four biological replicates per experiment.

For pCTgfp transmission in B104 strains, transmission assays were set up exactly as described above, with different donor/recipient ratios tested. Antiplasmid compounds were tested at the following concentrations: 3.5 mg/ml ascorbic acid, 6 mM linoleic acid, and 20 μg/ml chlorpromazine.

Details of how conjugation frequencies were determined can be found in Text S1.
Determining frequency of mutation for AZT resistance and selection of AZT-resistant mutants. To determine AZT frequency of mutation, ST131c, ST131 pCTgfp, and ST131 mcherry were plated onto LB agar plates containing 16 μg/ml AZT and LB agar plates containing no drug. Plates were incubated for 24 h at 37°C, and the colonies were enumerated. The Don Whitley Scientific automated spiral plater was used, per the manufacturer’s instructions. For each strain, 4 biological and 5 technical replicates were used. Mutation frequency was calculated by dividing the number of mutants by the viable count. From the AZT plates, mutant colonies were selected, and the MIC of AZT was determined as indicated above. Candidates with an AZT MIC of ≥32 μg/ml were selected, and the thymidine kinase gene was sequenced to determine if mutations had occurred (primers listed in Table S3). Mutants with deletions in thymidine kinase were then used in the AZT flow cytometry assay.

Fluorescent microscopy. Multichannel confocal images were taken at the Birmingham Light Advance Microscopy (BALM) facility at the University of Birmingham. In order to allow a proper comparison between different samples, the same optical configuration (digital zoom, photomultiplier tube (PMT), laser power, Galvano scanner, and pinhole) was used in all acquisitions. Samples were mixed to a final volume of 6 μl in morpholinepropanesulfonic acid (MOPS) minimal medium alone or with ascorbic acid (3.5 mg/ml), linoleic acid (6 μM), or chlorpromazine (50 μg/ml). A higher chlorpromazine concentration was used because of the short duration of this experiment. Bacteria were mounted on a conventional glass slide, placed on a heated stage (37°C), and immediately imaged every 10 min over 120 min. Time-lapse acquisition was performed sequentially by using the 488-nm and the 543-nm laser lines on a Zeiss LSM710 Confocor3 point scanning confocal system mounted on an Axio Observer inverted microscope with a 63×/1.4 oil objective (Zeiss).

Image analysis was performed on the open-source software ICY (http://icy.bioimageanalysis.org/) using the Wavelet Spot Detector and the Colocalization Studio plugins to assess the level of colocalization between the two different cells over time. A total of 12 images across 3 replicates for each time point and condition were analyzed. The number of colocalization events was normalized to the total number of bacteria and plotted using GraphPad Prism.

High-throughput screening. The Prestwick Chemical Library, containing 1,280 drugs and compounds, was tested in 96-well format using a Hamilton Star robot to identify compounds which inhibited the transmission of pCTgfp in ST131c or pKpQIL in K. pneumoniae. Compounds were dissolved in DMSO. A final concentration of 10 μM compound was added to the assay plate containing LB broth and a 3:1 or 1:2 mixture of donor and recipient cells (as described above). The final concentration of DMSO was tested and had no impact on bacterial growth or plasmid transmission. Plates were incubated for 24 h and read on the Attune flow cytometer as described above. The OD600 of each plate was determined using a FLUOstar Optima, as described above. Each compound plate was repeated in two independent experiments. Plates containing promising compounds were repeated a third time. The number of transconjugants in each well was determined and compared with LB controls. Compounds with levels of transmission below LB values were considered possible hits.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.04 MB.
FIG S1, DOCX file, 2 MB.
FIG S2, DOCX file, 2.3 MB.
FIG S3, DOCX file, 1.9 MB.
TABLE S1, DOCX file, 0.03 MB.
TABLE S2, DOCX file, 0.01 MB.
TABLE S3, DOCX file, 0.03 MB.
TABLE S4, XLSX file, 0.01 MB.
TABLE S5, XLSX file, 0.02 MB.
TABLE S6, XLSX file, 0.01 MB.

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M.M.C.B. and L.J.V.P. conceived the project and designed the experiments. M.M.C.B., M.L.C., R.W.M., A.R.M., E.L.P., G.E.M., J.P.R., L.J.A., and A.D.M. carried out the experiments and performed the data analysis. M.M.C.B., R.W.M., and L.J.V.P. contributed to writing. L.J.V.P. oversaw the work.

The authors declare no competing interests.
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