# Inhibiting plasmid mobility: The effect of isothiocyanates on bacterial conjugation

Awo Afi Kwapong [a,b], Paul Stapleton [a], Simon Gibbons [a,*]

[a] Research Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, University College London, 29–39 Brunswick Square, London WC1N 1AX, UK
[b] Department of Pharmaceutics and Microbiology, School of Pharmacy, University of Ghana, Accra, Ghana

### ABSTRACT

Bacterial conjugation is the main mechanism for the transfer of multiple antimicrobial resistance genes among pathogenic micro-organisms. This process may be controlled by compounds that inhibit bacterial conjugation. In this study, the effects of allyl isothiocyanate, ʟ-sulforaphane, benzyl isothiocyanate, phenylethyl isothiocyanate and 4-methoxyphenyl isothiocyanate on the conjugation of broad-host-range plasmids harbouring various antimicrobial resistance genes in *Escherichia coli* were investigated, namely plasmids pKM101 (IncN), TP114 (IncI$_2$), pUB307 (IncP) and the low-copy-number plasmid R7K (IncW). Benzyl isothiocyanate (32 mg/L) significantly reduced conjugal transfer of pKM101, TP114 and pUB307 to $0.3 \pm 0.6\%$, $10.7 \pm 3.3\%$ and $6.5 \pm 1.0\%$, respectively. ʟ-sulforaphane (16 mg/L; transfer frequency $21.5 \pm 5.1\%$) and 4-methoxyphenyl isothiocyanate (100 mg/L; transfer frequency $5.2 \pm 2.8\%$) were the only compounds showing anti-conjugal specificity by actively reducing the transfer of R7K and pUB307, respectively.

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## 1. Introduction

Bacterial conjugation is an adaptive mechanism that allows bacteria to transfer genetic material, effector proteins and/or toxins from one cell to another through a conjugative bridge [1,2]. The genetic material that is transferred via conjugation usually confers a selective advantage to the recipient organism, such as survival, resistance, pathogenicity, infection activities and/or the ability to respond to environmental changes. Conjugation greatly increases bacterial genome plasticity and has immense clinical relevance as a major route for the spread of multiple antimicrobial resistance genes among the microbial community and virulence genes from pathogen to host bacterium [2]. It is therefore imperative to find ways to combat conjugation as a means to decrease the ongoing rise of antimicrobial-resistant infections.

Inhibition of bacterial conjugation has received little research attention because the focus has been on the identification of new classes of antimicrobial agents that target processes essential for bacterial growth such as cell wall biosynthesis, the cell membrane, protein synthesis, nucleic acid synthesis and metabolic activity. This traditional approach has produced many therapeutically useful agents so far, but the challenge is that an antibiotic also introduces selective pressure promoting resistant bacteria and this has led to the current antibiotic resistance crisis. An additional approach for reducing the increasing rate of bacterial antimicrobial resistance dissemination and re-sensitising bacteria to existing antibiotics would be to target non-essential processes such as conjugation, which are less likely to evoke bacterial resistance. This approach could also have a prophylactic use in cosmeceuticals to reduce plasmid transfer. In addition to bacterial conjugation, other non-essential processes such as plasmid replication [3–5] and plasmid-encoded toxin–antitoxin systems [6,7] have been exploited with promising potential in antibacterial therapy.

The few efforts directed towards identifying anti-conjugants include small-molecule inhibitors of *Helicobacter pylori cag* VirB11-type ATPase Cagα [8]. The *cag* genes encode assembly of the conjugative bridge and injection of the CagA toxin into host cells [8,9]. In addition, there have been other reports of promising anti-conjugants, such as dehydrocrepenynic acid [1], linoleic acid [1], 2-hexadecyanoic acid [10], 2-octadecynoic acid [10] and tanzawaic acids A and B [11]. However, these compounds have stability, toxicity or scarcity issues that need to be addressed. Therefore, there is a pressing need to identify safer anti-conjugants to help in the fight against plasmid-mediated transfer and the spread of antimicrobial resistance and virulence.

In this study, four naturally occurring isothiocyanates [allyl isothiocyanate (**1**), ʟ-sulforaphane (**2**), benzyl isothiocyanate (**3**)

* Corresponding author. Tel.: +44 207 753 5913.
*E-mail address:* simon.gibbons@ucl.ac.uk (S. Gibbons).

and phenylethyl isothiocyanate (**4**)] as well as a synthetic isothiocyanate [4-methoxyphenyl isothiocyanate (**5**)] were investigated for their anti-conjugant activity against *Escherichia coli* strains bearing conjugative plasmids with specific antimicrobial resistance genes. Isothiocyanates are usually naturally occurring hydrolytic products of glucosinolates that are commonly found in *Brassica* vegetables. They are produced when damaged plant tissue releases the glycoprotein enzyme myrosinase, which hydrolyses the $\beta$-glucosyl moiety of a glucosinolate. This leaves the unstable aglycone thiohydroxamate-*O*-sulfonate, which rearranges to form an isothiocyanate or other breakdown products [12,13]. Other isothiocyanates, such as 4-methoxyphenyl isothiocyanate and methyl isothiocyanate, are synthetically produced and are not naturally occurring.

In addition to anti-conjugant testing, plasmid-curing activity and bacterial growth inhibition were also evaluated to help discriminate between true anti-conjugants and substances that reduce conjugation owing to elimination of plasmids or function by perturbation of bacterial growth or physiology. Isothiocyanates possessing the highest anti-conjugant activities were further investigated for cytotoxicity against human dermal fibroblasts, adult cells (HDFa; C-013-5C).

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*Escherichia coli* NCTC 10418 (a susceptible Gram-negative strain), *Staphylococcus aureus* ATCC 25923 (a susceptible Gram-positive strain), *S. aureus* SA-1199B (a fluoroquinolone-resistant strain that overexpresses the multidrug resistance NorA pump) and *S. aureus* XU212 (a tetracycline-resistant strain that overexpresses the multidrug resistance TetK pump) were used for the broth dilution assay. Plasmid-containing *E. coli* strains WP2, K12 J53-2 and K12 JD173 were used as donor strains in the plate conjugation and plasmid elimination assays. *Escherichia coli* ER1793 (streptomycin-resistant) and *E. coli* JM109 (nalidixic-resistant) were used as recipient strains. The conjugative plasmids used were pKM101 [WP2; incompatibility group N (IncN); ampicillin-resistant], TP114 (K12 J53-2; IncI$_2$; kanamycin-resistant) and R7K (K12 J53-2; IncW; ampicillin-, streptomycin- and spectinomycin-resistant), purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and conjugative plasmid pUB307 (K12 JD173; IncP; ampicillin-, kanamycin- and tetracycline-resistant), provided by Prof. Keith Derbyshire (Wadsworth Center, New York Department of Health, New York, NY).

### 2.2. Broth microdilution assay

Antibacterial activity was determined by the broth microdilution assay as described previously [14], which is a modified version of the procedure described in the British Society for Antimicrobial Chemotherapy (BSAC) guide to susceptibly testing [15]. Bacteria were cultured on nutrient agar slants and were incubated at 37 °C for 18 h. A bacterial suspension equivalent to a 0.5 McFarland standard was made from the overnight culture. This was added to Muller–Hinton broth and the test isothiocyanate, which had been serially diluted across a 96-well microtitre plate to achieve a final inoculum of $0.5 \times 10^5$ CFU/mL. Minimum inhibitory concentrations (MICs) were determined following 18 h of incubation at 37 °C. This was done by visual inspection after the addition of a 1 mg/mL methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and incubation at 37 °C for 20 min. This experiment was performed in duplicate in two independent experiments.

### 2.3. Liquid conjugation assay

Donor cells with plasmids pKM101, TP114 and pUB307 were paired with the recipient ER1793. Plasmid R7K donor cells were paired with the recipient JM109. Research has shown that plasmid carriage by host bacteria is associated with some fitness cost (burden) [16,17]. This fitness effect of plasmids plays a vital role in their ability to associate with a new bacterial host. As a consequence of this, different *E. coli* hosts that are known to successfully conjugate [18,19] and to maintain the study plasmids were selected. The liquid conjugation assay was performed as previously described [20] with slight modifications. Equal volumes (20 μL) of donor and recipient cells, for which the CFU/mL had been predetermined (Supplementary Table S1), were introduced into 160 μL of Luria–Bertani broth and the test sample or control. This was incubated at 37 °C for 18 h, after which the number of transconjugants and donor cells was determined using antibiotic-containing MacConkey agar plates. A positive control (linoleic acid [1]) and a negative control (donor, recipient and medium, without drug or test sample) were included in the experiment. The isothiocyanates were evaluated for anti-conjugant activity at a subinhibitory concentration ($0.25 \times$ MIC). Antibiotics were added at the following concentrations for positive identification of donors, recipients and transconjugants: amoxicillin (30 mg/L); streptomycin sulphate (20 mg/L); nalidixic acid (30 mg/L); and kanamycin sulphate (30 mg/L). Conjugation frequencies were calculated as the ratio of total number of transconjugants (CFU/mL) to the total number of donor cells (CFU/mL) and were expressed as a percentage relative to the negative control. This experiment was performed in duplicate in three independent experiments and the anti-conjugation activity was reported as the mean $\pm$ standard deviation (S.D.).

### 2.4. Plasmid elimination assay

The plasmid elimination assay was performed as described previously [21] with minor modifications. *Escherichia coli* donor strains were subcultured on appropriate antibiotic-containing MacConkey agar plates to ensure plasmid presence. Following incubation of the plates at 37 °C for 18 h, two to three single colonies were selected and were inoculated into Luria–Bertani broth. This was incubated for 18 h at 37 °C and the CFU were determined prior to the assay. Then, 20 μL of the overnight culture was added to a mixture of 180 μL of LB and test sample in a 96-well microtitre plate. This was incubated overnight (18 h) at 37 °C and was subsequently serially diluted, then 20 μL was plated on antibiotic-containing MacConkey agar and was incubated for 18 h at 37 °C. The isothiocyanates were evaluated for plasmid elimination activity at concentrations used in the liquid conjugation assay. Both a positive control (promethazine) [22–24] and negative control (mixture without isothiocyanate or control drug) were included in this experiment. Plasmid elimination was calculated using the equation:

$$\text{Plasmid elimination} = \frac{\text{CFU/mL of control} - \text{CFU/mL of test sample}}{\text{CFU/mL of control}} \times 100$$

Antibiotics and concentrations used in MacConkey agar for positive identification of *E. coli* cells harbouring plasmids were amoxicillin (30 mg/L), kanamycin sulphate (20 mg/L and 30 mg/L) and nalidixic acid (30 mg/L). This experiment was performed in duplicate with three independent experiments.

### 2.5. Cytotoxicity assay

The isothiocyanates showing anti-conjugant activity were further assessed for their effect on eukaryotic cell growth. The sulforhodamine B (SRB) colorimetric assay was used as described

previously [25] with modifications. Human dermal fibroblasts, adult cells (HDFa; C-013-5C) were grown in a 75-cm$^2$ culture flask at 37 °C in a humidified atmosphere of 5% carbon dioxide using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 0.1% gentamicin and amphotericin B. The grown cells were seeded in a 96-well microtitre plate and the test samples and medium were added. The plates were then incubated at 37 °C in 5% CO$_2$ for 72 h. Then, 50 µL of cold 40% w/v trichloroacetic acid solution was added, the plate was placed in the fridge for 1 h at 4 °C and was washed four times with distilled water. Cells were then stained with 0.4% w/v SRB solution and were left at room temperature for 1 h. The plate was then rinsed four times with 1% acetic acid and was left overnight (24 h) to dry. Thereafter, 100 µL of 10 mM Tris buffer solution was dispensed into the wells and was agitated in an orbital shaker for 5 min to allow solubilisation of SRB–protein complexes. The optical density (OD) at 510 nm was then measured using a microtitre plate reader (Tecan Infinite® M200; Tecan Life Sciences, Grödig, Austria). The percentage of viable cells was calculated using the equation:

$$\text{Percentage of viable cell} = \frac{\text{OD of test sample} - \text{OD of blank}}{\text{OD of negative control} - \text{OD of blank}} \times 100$$

The experiment was performed as triplicate in three independent experiments, and cytotoxicity was reported as the mean ± S.D.

### 2.6. Statistical analyses

Statistical analyses were carried out using Excel Data Analysis (Microsoft Corp., Redmond, WA) and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). Welch's *t*-test was used to evaluate differences between the control conjugal transfer frequency and the test compounds. Results with *P*-value of <0.05 were considered statistically significant.

## 3. Results

### 3.1. Effect of isothiocyanates on bacterial growth

To test whether the selected isothiocyanates had growth-inhibitory activity against bacterial species and to determine a suitable concentration for their evaluation in the anti-conjugation assay, the isothiocyanates were tested against susceptible Gram-negative (*E. coli* NCTC 10418) and Gram-positive (*S. aureus* ATCC 25923) standard isolates as well as antibiotic-effluxing *S. aureus* strains (SA-1199B and XU212). Table 1 shows the MICs for the tested isothiocyanates. Their inhibitory activity varied from 16 mg/L to >512 mg/L against the evaluated bacteria. The general observation was that, unsurprisingly, the isothiocyanates were marginally more active against the Gram-positive compared with the Gram-negative strains.

### 3.2. Effect of isothiocyanates on conjugal transfer of plasmids

To investigate whether the selected isothiocyanates had anti-conjugant activity, a range of plasmids belonging to different incompatibility groups (IncN plasmid pKM101, IncI$_2$ plasmid TP114, IncP plasmid pUB307 and IncW plasmid R7K) were employed to test the specificity of conjugation inhibition in *E. coli*. With information about their MIC against *E. coli* NCTC 10418 (a susceptible standard strain) (Table 1), the isothiocyanates were tested at a subinhibitory concentration (0.25× MIC). Fig. 1 shows the effect of the isothiocyanates on conjugal transfer of the test plasmids. The test isothiocyanates exhibited inhibitory activities ranging from a complete reduction in conjugation frequency (0%, considered active) and inhibition of conjugation frequency to <10% (also considered active), to 10–50% (considered moderately active) and >50% (considered inactive) (Supplementary Table S2).

### 3.3. Elimination of plasmids from Escherichia coli

To determine that the observed anti-conjugant activity was not due to elimination of conjugative plasmids, donor cells were grown in the presence of the test isothiocyanates and the plasmid elimination assay was performed. Fig. 2 shows the effect of the isothiocyanates on conjugative plasmids. The isothiocyanates exhibited varied plasmid-curing activities. Plasmids TP114 (IncI$_2$) and R7K (IncW) were the most eliminated in the donor cells, with elimination percentages ranging from 3.0 ± 0.1% to 77.8 ± 8.0%. Most of the tested isothiocyanates did not have any plasmid-curing effects on pKM101 (IncN), with the exception of allyl isothiocyanate (**1**), which showed a curing effect of 19.4 ± 6.6%. For pUB307 (IncP), a plasmid-curing effect was observed for ʟ-sulforaphane (**2**) (56.7 ± 3.2%) and phenylethyl isothiocyanate (**4**) (64.8 ± 15.4%) (Supplementary Table S3).

### 3.4. Effect of increasing concentrations of benzyl isothiocyanate (3) on conjugal transfer of plasmids pKM101 (IncN), TP114 (IncI$_2$) and pUB307 (IncP)

With benzyl isothiocyanate (**3**) having shown broad-range anti-conjugant (conjugal reduction to 0.3 ± 0.6–10.7 ± 3.3%; Fig. 1) and the least donor plasmid elimination activity (0–26.5 ± 5.9%; Fig. 2) of all tested compounds, it was further assessed to observe its effect on conjugal transfer at increasing concentrations. Generally, there was a gradual increase in anti-conjugal activity against pKM101 and TP114 with an increase in concentration from 0.125 mg/L to 64 mg/L (Fig. 3), whereas for plasmid pUB307 there was no significant change in the anti-conjugal activity for benzyl isothiocyanate (**3**) and it surprisingly remained active at the low concentrations tested. The observed conjugal transfer of pUB307 in the presence of **3** ranged from 11.3 ± 2.6% to 1.9 ± 2.2% for concentrations of 0.125 mg/L and 64 mg/L, respectively (Supplementary Table S4).
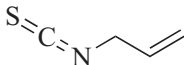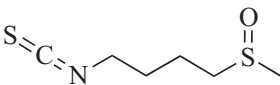
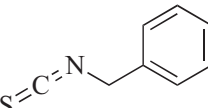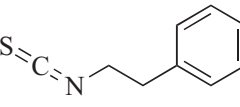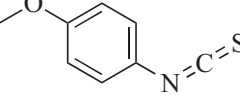### 3.5. Effect of increasing concentrations of 4-methoxyphenyl isothiocyanate (5) on conjugal transfer of pUB307

Among the test isothiocyanates, 4-methoxyphenyl isothiocyanate (**5**) was the most active against plasmid pUB307 (IncP) with no plasmid-curing activity. It was therefore evaluated for the effect of increasing concentrations (1–128 mg/L) on the conjugal transfer of plasmid pUB307. The observed activities are shown in Fig. 4. 4-Methoxyphenyl isothiocyanate (**5**) showed a moderate anti-conjugant activity (22.7 ± 1.6%) at the lowest concentration (1 mg/L) and this was steadily maintained up to 32 mg/L, after which there was a sharp increase in conjugal inhibition. Almost complete conjugal inhibition was observed at 128 mg/L (Supplementary Table S5).

### 3.6. Effect of allyl (1) and benzyl (3) isothiocyanates on normal growth of human dermal fibroblasts, adult cells (HDFa; C-013-5C)

Allyl (**1**) and benzyl (**3**) isothiocyanates that exhibited active to moderate anti-conjugant activity against all test plasmids were further assessed for cytotoxicity against normal cell growth. This was to determine whether the broad-range anti-conjugant activities exhibited by isothiocyanates **1** and **3** were not at cytotoxic concentrations and thus worth pursuing as potential anti-conjugants for further development. The observed cytotoxic activities are shown

**Table 1**
Minimum inhibitory concentrations (MICs) of isothiocyanates and comparators against *Escherichia coli* and *Staphylococcus aureus* strains.

| Isothiocyanate | Chemical structure | MIC (mg/L) | | | |
|---|---|---|---|---|---|
| | | *E. coli* NCTC 10418[a] | *S. aureus* ATCC 25923[a] | *S. aureus* SA-1199B[b] | *S. aureus* XU212[c] |
| Allyl isothiocyanate (**1**) | | > 512 | 512 | 512 | > 512 |
| L-sulforaphane (**2**) | | 64 | 64 | 32 | 32 |
| Benzyl isothiocyanate (**3**) | | 128 | 256 | 256 | 512 |
| Phenylethyl isothiocyanate (**4**) | | 256 | 16 | 32 | 32 |
| 4-Methoxyphenyl isothiocyanate (**5**) | | 512 | 256 | 128 | 128 |
| Ciprofloxacin | – | <0.0625 | <0.0625 | – | – |
| Norfloxacin | – | – | – | 32 | – |
| Tetracycline | – | – | – | – | 128 |

[a] Susceptible standard strains.
[b] Fluoroquinolone-resistant strain overexpressing the NorA efflux pump.
[c] Tetracycline-resistant strain overexpressing the TetK efflux pump.
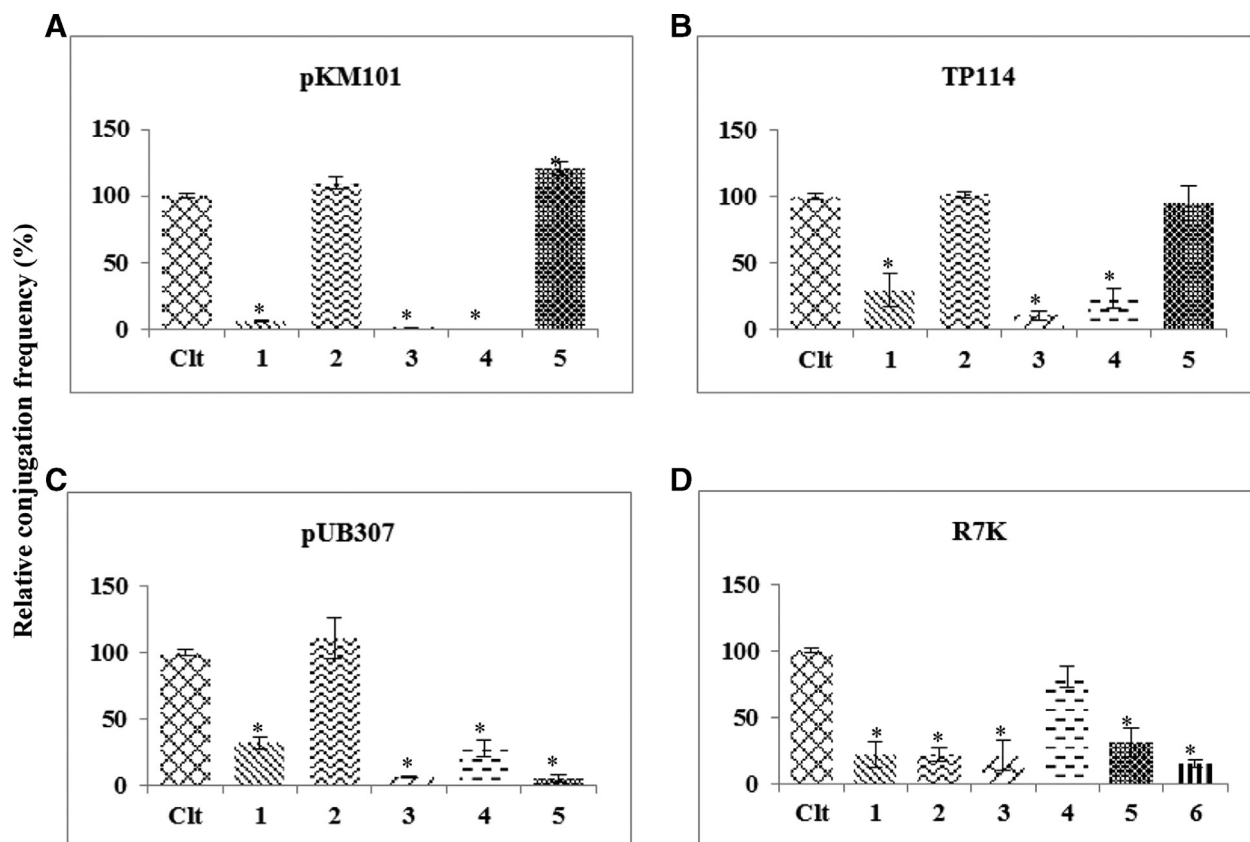


**Fig. 1.** Effect of selected isothiocyanates on conjugal transfer of (A) IncN plasmid pKM101, (B) IncI₂ plasmid TP114, (C) IncP plasmid pUB307 and (D) IncW plasmid R7K, expressed as a percentage relative to the control without test compound (Clt). The isothiocyanates were tested at the following subinhibitory concentrations: allyl isothiocyanate (**1**) (100 mg/L); L-sulforaphane (**2**) (16 mg/L); benzyl isothiocyanate (**3**) (32 mg/L); phenylethyl isothiocyanate (**4**) (64 mg/L); and 4-methoxyphenyl isothiocyanate (**5**) (100 mg/L). Linoleic acid (**6**), a known anti-conjugant for IncW plasmids, was tested at 200 mg/L. Values represent the mean ± standard deviation of at least three independent experiments measured by the plate conjugation assay. * $P < 0.05$ (compared with the control).
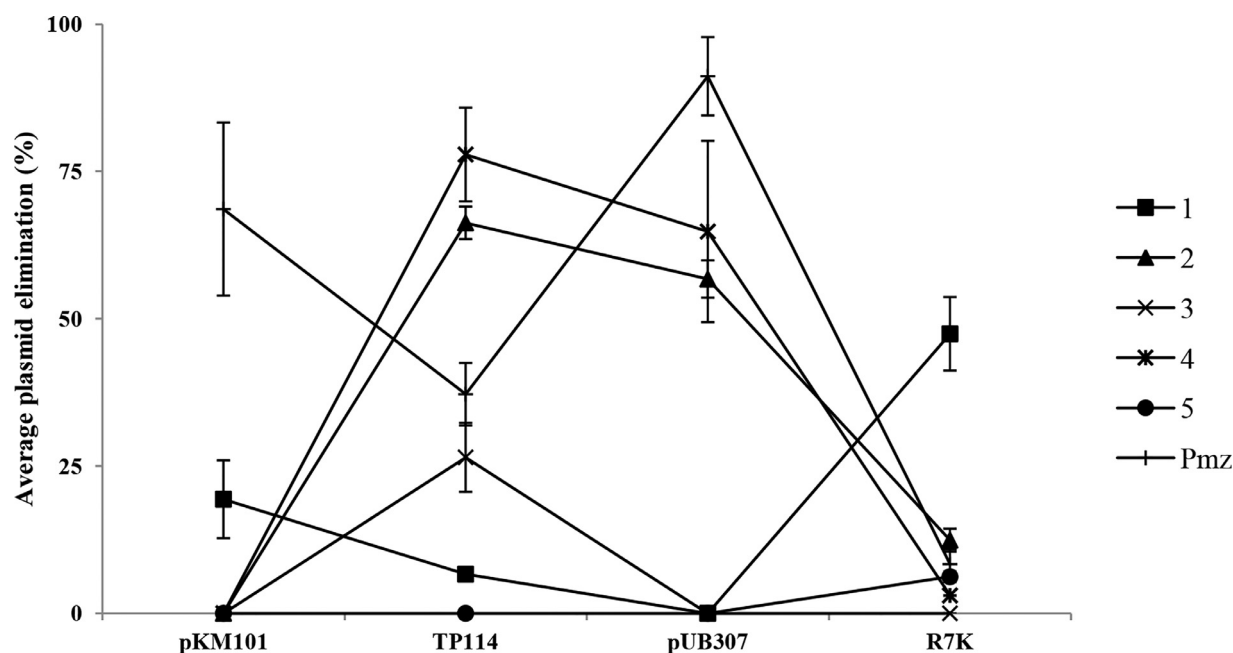
**Fig. 2.** Plasmid elimination activity of the isothiocyanates. The isothiocyanates were tested at the following concentrations: allyl isothiocyanate (**1**) (100 mg/L); L-sulforaphane (**2**) (16 mg/L); benzyl isothiocyanate (**3**) (32 mg/L); phenylethyl isothiocyanate (**4**) (64 mg/L); 4-methoxyphenyl isothiocyanate (**5**) (100 mg/L); and promethazine (Pmz) (16 mg/L).
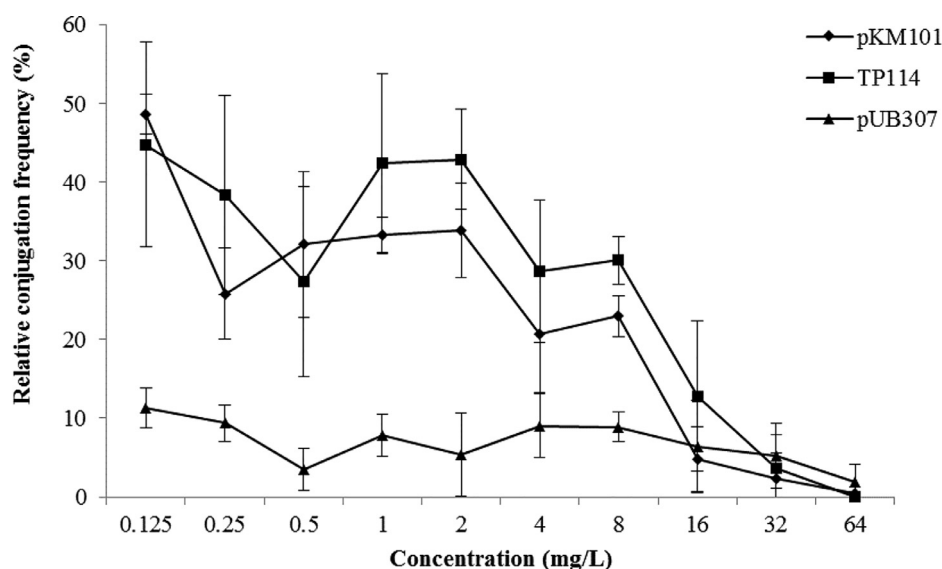


**Fig. 3.** Effect of increasing concentrations of benzyl isothiocyanate (**3**) on conjugal transfer of plasmids pKM101 (IncN), TP114 (IncI$_2$) and pUB307 (IncP) relative to the control without test sample (100% conjugation frequency). Values represent the mean ± standard deviation of a least three independent experiments measured by the plate conjugation assay.

in Fig. 5. The 50% cytotoxic concentration (CC$_{50}$) values for allyl (**1**) and benzyl (**3**) isothiocyanates against HDFa cells were 63.9 mg/L (645 μM) and 30.3 mg/L (203 μM), respectively (Supplementary Table S6).

## 4. Discussion

The discovery of a potent compound that will inhibit the spread of resistance genes and/or resistance mechanisms has clinical relevance, especially in this era of plasmids in species such as *Klebsiella pneumoniae* that are carbapenem-resistant. This is highly timely given the lack of treatment options for infections caused by this pathogen. In line with this, selected isothiocyanates, which are hydrolysis products of glucosinolates commonly found in *Brassica*

vegetables, were investigated for the possibility of inhibiting the spread of resistance genes by blocking bacterial conjugation in *E. coli*.

The initial findings from this study showed that allyl isothiocyanate (**1**), L-sulforaphane (**2**), benzyl isothiocyanate (**3**), phenylethyl isothiocyanate (**4**) and 4-methoxyphenyl isothiocyanate (**5**) have some level of antibacterial activity ranging from 16 mg/L to >512 mg/L against susceptible *E. coli* NCTC 10418 and *S. aureus* ATCC 23925 as well as effluxing multidrug-resistant *S. aureus* strains (SA-1199B and XU212) (Table 1). This corroborates the reported antibacterial activity of the isothiocyanates but, owing to the variability in testing methods, bacterial inoculum densities and diversity in susceptibility, it is difficult to compare results [26–32]. The isothiocyanates were found to be less potent compared with
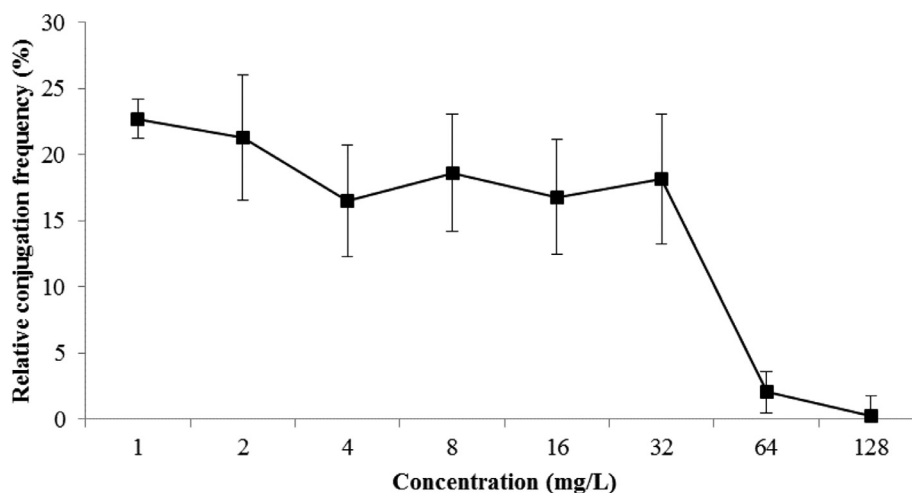
**Fig. 4.** Effect of increasing concentrations of 4-methoxyphenyl isothiocyanate (**5**) on conjugal transfer of IncP plasmid pUB307 relative to the control without test sample (100% conjugation frequency). Values represent the mean ± standard deviation of a least three independent experiments measured by the plate conjugation assay.
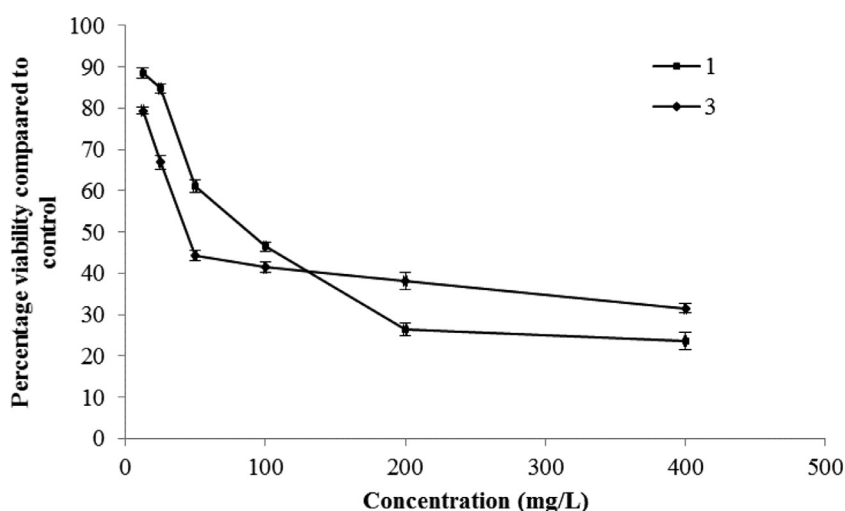


**Fig. 5.** Effect of allyl (**1**) and benzyl (**3**) isothiocyanates on growth of human dermal fibroblasts, adult cells (HDFa; C-013-5C). Values represent the mean ± standard deviation of a least three independent experiments measured by cytotoxicity assay.

conventional antibiotics, and similar results have been reported by others [26,28,32]. Among the tested isothiocyanates, phenylethyl isothiocyanate (**4**) was the most potent against Gram-positive microbes with MICs ranging from 16 mg/L to 32 mg/L, followed by L-sulforaphane (**2**) (MIC range 32–64 mg/L), which was also the most potent against Gram-negative *E. coli* NCTC 10418. The antibacterial activities of these isothiocyanates have been explained to be due to their ability to cause physical membrane damage [33,34], to interfere with the bacterial redox system that affects the cell membrane potential [34], or disruption of major metabolic processes [35,36].

Regarding the anti-conjugal activity study, broad-range anti-conjugant activity was observed for allyl (**1**) and benzyl (**3**) isothiocyanates at subinhibitory concentrations, with **3** being the most potent among the tested isothiocyanates (Fig. 1). It inhibited the conjugation of plasmids pKM101 (IncN), TP114 (IncI$_2$) and pUB307 (IncP), and selectively cured plasmid TP114 only. Against plasmids pKM101 and TP114, **3** also reduced conjugal transfer by 97.7 ± 3.3% and 96.4 ± 4.2%, respectively, at 32 mg/L (214.46 μM) and its activity gradually declined with decreasing concentrations (Fig. 3). This was not the same for pUB307, where **3** continued to show pronounced activity with a 90.8 ± 2.3% reduction in conjugation even at a low concentration of 0.25 mg/L (1.68 μM). This was interesting as **3** did not show any plasmid-curing activity against this particular plasmid pUB307 or against pKM101, ruling out the fact that the observed anti-conjugation may be due to plasmid elimination. Another area of interest was that **3** exhibited broad-range activity; this could mean that **3** either acts on a common target site on the conjugation machinery or that it causes general cell toxicity. However, considering the MIC (128 mg/L; Table 1) of **3** against the susceptible *E. coli* strain NCTC 10418, the concentrations ($\leq$32 mg/L) used for the conjugation assays were at sublethal doses and are less likely to have caused general cell toxicity. With allyl isothiocyanate (**1**), moderate plasmid elimination activity was observed against most of the test plasmids and this may be an indication that its broad-range anti-conjugant activity is due to plasmid curing. The broad-range activities of **1** and **3** prompted their testing against normal growth of human dermal fibroblasts, adult cells (HDFa; C-013-5C). A comparison of the CC$_{50}$ of **3** against HDFa cells (30.30 mg/L; 203.07 μM) with its anti-conjugant concentration against the test plasmids showed that its CC$_{50}$ level was above the concentrations needed to cause a 50% reduction in conjugal transfer of plasmids; pKM101 (CC$_{50}$ = 2.19 mg/L; 14.68 μM); TP114 (CC$_{50}$ = 1.24 mg/L; 8.31 μM); and pUB307 (CC$_{50}$ = 0.34 mg/L; 2.28 μM) (Fig. 5). This suggests that **3** showed anti-conjugant activity at non-toxic concentrations. However, the same cannot be said for allyl isothiocyanate (**1**) because its CC$_{50}$ against HDFa cells

(63.9 mg/L; 644.48 μM) was below the 100 mg/L needed to cause moderate anti-conjugant activity (50–90% reduction) against most of the test plasmids. It is therefore suggested that the concentrations needed to cause a 50% reduction in conjugation is most likely to be closer to the $CC_{50}$ value.

From this study, specificity of anti-conjugal and plasmid-curing activity was observed for 4-methoxyphenyl isothiocyanate (**5**), a synthetic compound. L-sulforaphane (**2**) also exhibited some level of anti-conjugant specificity against the IncW plasmid R7K at 16 mg/L (90.25 μM), but at this same concentration plasmid curing was observed and hence **2** is not a true anti-conjugant (Fig. 2). Anti-conjugant activity of **5** at 100 mg/L (605.29 μM) was pronounced for the IncP plasmid pUB307, with a 94.8 ± 2.8% reduction in conjugation, but it showed minimal inhibition or even promoted conjugation for the other test plasmids (Fig. 1). Its anti-conjugant activity was, however, concentration-dependent (Fig. 4). Regarding the plasmid-curing effect, **5** showed elimination of only the IncW plasmid R7K but it did not have any effect on conjugation of this plasmid. This may give an indication that **5** could have some conjugation promotion factors, and this was observed for pKM101. Conjugation of pKM101 in the presence of **5** exceeded 100% (Fig. 1). The anti-conjugation, plasmid-curing and pro-conjugation activities exhibited by **5** supports its specificity. This suggests that compound **5** acts on a specific target site that may not be common to all plasmids. Consequently, it is less likely for resistance to develop against **5**, unlike other compounds that target general and essential targets of bacteria, which is the case in many instances of antibiotic resistance [37]. A general observation with the test isothiocyanates is that the presence of oxygen, attached to sulphur or an aromatic carbon, conferred some level of anti-conjugal specificity. We therefore hypothesise that the methoxyl substituent on the aromatic ring and the lack of a hydrocarbon chain of **5**, which makes it structurally different from the other test aromatic isothiocyanates, may have contributed to its specificity of activity.

In conclusion, isothiocyanates **3** and **5** were the most promising anti-conjugants identified in this study. Further explorative studies involving structural modification and mechanistic studies of these isothiocyanates could possibly lead to the identification of a potent anti-conjugant. This will help decrease the spread of multidrug resistance genes and multidrug-resistant bacteria, reduce virulence and help reinstate existing antibiotics.

## Funding

## Competing interests

None declared.

## Ethical approval

Not required.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.01.011.

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