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Elimination of Plasmids by SILA Compounds that Inhibit Efflux Pumps of Bacteria and Cancer Cells

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Abstract. *Patented SILA compounds 409 and 421, previously shown to inhibit the efflux pumps of bacteria and cancer cells, have been studied for their ability to reduce or eliminate the presence of plasmids from Escherichia coli strains that have been induced to high level resistance to tetracycline by gradual exposure to increasing concentrations of the antibiotic. The results demonstrate that SILA compound 421, which has greater efflux pump inhibitory activity than its parent SILA compound 409, can reduce plasmid loads by 5 logs, over that present in the absence of the drug. The ability of the SILA compound to eliminate much larger plasmids is substantially lower. Because in vivo studies have shown that these compounds are not toxic to the mouse, the results obtained in our study suggest a potential role for SILA compound 421 as an adjunct for the therapy of antibiotic-resistant E. coli infections whose resistance is plasmid-mediated. In addition, because plasmid-mediated resistance is often found in tetracycline-treated cattle, SILA compound 421 may have potential as an adjunct during the time that the cattle are maintained on tetracycline prior to slaughter.*

Plasmid-mediated antibiotic resistance of Gram-negative bacteria is common in human infections (1-5) and despite the use of tetracycline (TET) it is now becoming quite common in cattle (6, 7). Plasmid-mediated resistance due to the production of plasmid-mediated AmpC beta-lactamases is especially difficult to treat inasmuch as inhibitors for these enzymes are still not available (8-10). For over 30 years, phenothiazines have been known to cause the elimination of plasmids (11-15). Nevertheless, because phenothiazines that produce *in vitro* effects produce them at concentrations which are well beyond those that can be achieved in clinical practice (15, 16), the use of these compounds for the elimination of plasmids that mediate antibiotic resistance in infections caused by Gram-negative bacteria has not been recommended. However, recent studies have shown that compounds that inhibit efflux pumps of bacteria can also reduce the plasmid load of Gram-negative bacteria (17). Because recently patented SILA compounds have been shown to inhibit the efflux pumps of Gram-negative bacteria (13, 17, 18), we have conducted studies that test the abilities of these SILA compounds to eliminate plasmids from *Escherichia coli* that overexpress efflux pumps.

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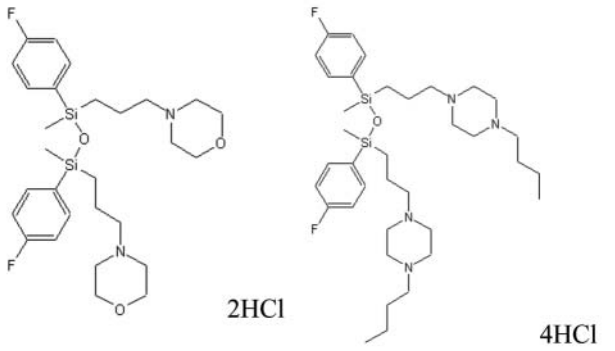
Materials and Methods

Bacteria. The bacteria employed in this study was *E. coli* AG100 acrAB (*argE3 thi-1 rpsL xyl mtl* Δ(*gal-uvrB*) supE44) (18) and *E. coli* F'lac K12 LE140 (*tsx, str, Δlac, su⁻, λr, mal⁻*) (12). *E. coli* AG100 *acrAB* strain was gradually exposed to increasing concentrations of TET until its resistance to TET increased from a minimum inhibitory concentration (MIC) of 2.0 to 12.0 mg/L. The strain induced to TET resistance was named *E. coli* $AG100_{TET}$ (18).

Materials. Tetracycline (TET) was purchased from Sigma (Madrid, Spain). Minimal-tryptone-yeast extract (MTY) nutrient broth, Trypticase Soy Broth (TSB) and Eosin Methylene-blue Lactose Sucrose Agar (EMB agar) were purchased from Difco (Detroit, Michigan, USA). Patented compounds 1,3-dimethyl-1,3-*bis* (4-fluorophenyl)-1,3-*bis*(3-morpholino-propyl)-disiloxan-dihydrochloride (SILA 409) and 1,3-dimethyl-1,3-*bis*(4-fluorophenyl)-1,3-*bis*{3-[1(4-buthyl-piperazinyl)]-propyl}-disiloxan-tetrahydrochloride (SILA 421) (Figure 1) were synthesised by Hegyes *et al.* (19). SILA compounds 409 and 421 have received patents, Brevet Europeen n0 0099150.6, PCT/DE00/04110.

Minimum inhibitory concentrations. The MIC of the two selected SILA compounds 409 and 421 against *E. coli* $AG100_{TET}$ was determined by the broth dilution method described elsewhere (20).

Reversal of resistance to TET. The evaluation of the two SILA compounds for their ability to reverse the resistance of the AG100TET TET induced resistant strain was conducted as follows:



Patent Brevet Europeen n0 0099150.6, PCT/DE00/04110

Figure 1. *Structure of patented compounds.*

SILA concentrations of 1, 10 and 50 mg/L were added to tubes containing 8 mg/L of TET in Trypticase Soy Broth (TSB), concentrations that have been shown not to appreciably affect the *in vitro* growth of the strain (18). The tubes were inoculated with 10 μL of a suspension adjusted to 0.5 McFarland scale, incubated at 37˚C for 24 h without shaking. Optical densities were recorded at 545 nm against the control without any SILA compound added and percent inhibition calculated.

The elimination of the metabolic plasmid of E. coli F'lac K12 LE140 by SILA compounds 409 and 421. An overnight pre-culture of *E. coli* F'lac K12 LE140 was diluted 10^4-fold in a saline solution and 0.05 μL aliquots (approximately $5x10^3$ cells) inoculated into 5.0 mL of minimal-tryptone-yeast extract (MTY) nutrient broth. Different concentrations of the SILA compounds 409 and 421 were added. In addition, because promethazine (PMZ) has been shown to cause the elimination of this plasmid from *E. coli* F'lac K12 LE140 (14), separate cultures containing different concentrations of this agent served as positive controls, as well as providing a basis for comparison. The cultures were incubated at 37˚C for 24 h without shaking. Two dilutions, with 10^4- and 10^5-fold concentrations, were prepared from tubes showing growth and plated in 100 μL amounts on eosin methylene-blue lactose sucrose agar (EMB agar). The plates were incubated at 37˚C for 24 h, and counted for *lac*$^-$ plasmidless (pink) and *lac*$^+$ plasmid-containing (deep-violet) colonies. The percentage of plasmid elimination was calculated and compared to the positive control provided by the PMZ containing cultures.

The pBR322 plasmid elimination activity of SILA compounds 409 and 421. E. coli AG100 (K12) strain (18) transformed with pBR322 (amp^r), was incubated for 24 h at 37˚C, without shaking, in TSB containing concentrations of compounds SILA 421 or SILA 409 at 25 mg/L, 50 mg/L and 100 mg/L. At the end of the incubation period, aliquots of 100 μL were transferred to 10 ml saline tubes and from these serial dilutions of 10^{-3} were made. From each serial dilution, duplicate aliquots of 100 μL were plated on ampicillin-free agar and ampicillin-containing agar (100 mg/L). After overnight incubation at 37˚C the colony forming units (CFU) present from a specific dilution that allowed fairly well separated CFU were counted. Whereas the presence of ampicillin in one set of plates allowed the growth of bacteria that contained the pBR322 plasmid, plates that contained no ampicillin would allow both plasmid-containing and plasmid-free bacteria to grow. The total number of CFU that were present in plates containing ampicillin therefore afforded an understanding of the degree of plasmid curing ability of SILA compounds on a concentration dependent basis. Deducting these CFU from the corresponding plates that contained no ampicillin afforded an understanding of the number of CFU that had been freed of plasmids. The data is presented as total log numbers reduced due to the presence of concentrations of SILA compound 409 and 421. The evaluation of SILA compounds 409 and 421 for *in vitro* toxicity was accomplished with the aid of the Trypan Blue Exclusion method described elsewhere (22).

Table I. *Evaluation of SILA compounds for their ability to reverse resistance to TET.*

	% Reversal of resistance to TET		
SILA compound	1 mg/L	10 mg/L	50 mg/L
409	21	34	46
421	10	77	98

SILA compounds at concentrations of 1, 10 and 50 mg/L were added to Trypticase Soy Broth (TSB) tubes containing 8 mg/L of TET and inoculated with *E. coli* $AG100_{TET}$ TET induced resistant strain (MIC of 12 mg/L) (18). After incubation at 37˚C for 24 h, without shaking, percent inhibition was calculated from optical densities recorded at 545 nm against the control without any SILA compound.

Results

The evaluation of SILA compound 409 and 421 activity against *E. coli* $AG100_{TET}$ induced to high level resistance to TET by the MIC broth dilution assay showed that a concentration as high as 100 mg/L of these compounds did not affect the growth of the strain (data not shown). The use of the Trypan Blue exclusion test showed that concentrations of SILA compounds as high as 100 mg/L did not cause more than 8% of the lymphocytes to stain with the dye, hence the compounds can be considered to be free of any toxicity to cells *in vitro* (22). Previous studies showed that SILA compound 409 had no toxic effect on the liver, lung or kidney of the mouse (23) and since SILA compound 421 is derived from its 409 parent, it was not surprising that it too would be devoid of toxicity at the cellular level. The evaluation of SILA compounds for their ability to reverse resistance to TET is presented in Table I. It is evident from this table that SILA compound 421 essentially reverses the resistance of the TET-induced strain at a concentration of 50 mg/L. SILA compound 409 is far less effective. The effects of SILA compounds on the elimination of the F'lac K12LE140 is summarised in Table II and the results may be compared to those obtained with the

Table II. Effect of SILA compounds 409 and 421 on plasmid elimination from E. coli F'lac K12 LE140.

Compound	Concentration (mg/L)	Plasmid elimination (%)
Control	-	2
PMZ	40	1.2
	50	1.6
	60	22.95
	80	57.2
	90	52.3
	100	3.95
	110	0
	120	0
SILA 409	20	0
	40	0.25
	80	17.75
	120	24.05
	160	39.025
	200	46.25
	240	27.65
SILA 421	20	0.2
	40	0.85
	80	52.375
	120	82.475
	160	72.15
	200	9.325
	240	0.0

Adjusted cultures of *E. coli* F'lac K12 LE140 inoculated tubes with 5.0 mL of MTY nutrient broth containing a range of concentrations of compounds 409, 421 and PMZ (control). The cultures were incubated at 37˚C for 24 h without shaking. 100 μL aliquots were plated on EMB agar for CFU counting after incubation at 37˚C for 24 h. *lac*$^-$ plasmidless colonies appeared as pink and *lac*$^+$ plasmid-containing colonies appeared as deep-violet and the percentage of plasmid elimination calculated. Concentrations of the SILA compounds above 160 mg/L showed to have severe effects on the growth of *E. coli*.

positive control PMZ. Whereas a sub-inhibitory concentration of PMZ of 80 mg/L (MIC>150 mg/L (24)) produced an elimination of plasmids of 57%, a far higher concentration of SILA compound 409 (200 mg/L) was required to produce an almost similar effect (46%). In contrast to the activity of SILA compound 409, SILA compound 421 at a concentration of 80 mg/L yielded a similar activity as that for the positive control PMZ, and at a concentration of 120 mg/L its plasmid elimination activity exceeded the maximum produced by PMZ. Comparing the plasmid elimination effectiveness of the SILA compounds, whereas the maximum effectiveness produced by SILA 409 took place at a concentration of 200 mg/L, the maximum produced by SILA 421 took place at a much lower concentration (120 mg/L).

The effects of SILA compounds on the elimination of the pBR322 plasmid from *E. coli* AG100TET induced is presented in Table III. Briefly, whereas the absence of the

Table III. *The effect of SILA compounds 409 and 421 on the elimination of pBR322 from E. coli.*

	CFU present on ampicillin-containing agar		
Control	10^8		
SILA compound	25 mg/L	50 mg/L	100 mg/L
409	10^8	10^7	10^5
421	10^7	10^5	10^3

TSB tubes containing concentrations of compounds SILA 421 or SILA 409 at 25 mg/L, 50 mg/L and 100 mg/L were inoculated with *E. coli* AG100 (K12) strain (18) transformed with pBR322 and incubated for 24 h at 37˚C, without shaking. Aliquots of 100 μL were then serial diluted and plated in duplicate aliquots on ampicillin-free agar and ampicillin-containing agar (100 mg/L). After overnight incubation at 37˚C the colony forming units (CFU) were counted and % of plasmid loss recorded.

compounds yielded approximately 108 CFU in the presence of ampicillin-containing agar, the presence of 50 and 100 mg/L of SILA compound 421 yielded approximately 10^5 and 10^3 CFU respectively on plates that contained ampicillin. These results show that SILA compound 421 can cause the elimination of plasmids by as much as 5 logs. The ability of SILA 409 to eliminate the pBR322 plasmid from *E. coli* was much lower.

Discussion

The results presented in this study show that SILA compounds that reverse induced resistance to TET can eliminate plasmids from *E. coli*. However, the type of plasmid employed for the evaluation of a compound for its ability to eliminate plasmids from plasmid-carrying *E. coli* appears to be extremely important given the comparative results obtained. Firstly, whereas the maximum elimination of the F'lac K12LE140 plasmid by the more effective SILA 421 compound was barely one log, that produced by that same SILA compound on the elimination of pBR322 was about 5 logs. The difference noted is probably due to the large difference in their size, since pBR322 is considerably smaller (12, 18).

Phenothiazines affect the physical (25, 26) and functional (27) properties/characteristics of the cell envelope of bacteria. At concentrations near their MIC they alter the electrophoretic pattern of proteins extracted from the cell envelope (28), cause its destruction (27) and inhibit influx/efflux transport mechanisms of the bacterium (18, 20, 29). The elimination of plasmids from *E. coli* by phenothiazines has been attributed to their differential effects on the synthesis of plasmid and bacterium DNA,

with greater effects noted on plasmid DNA synthesis (30). Because these compounds were shown in the current study to reverse the resistance to TET due to overexpressed efflux pumps, and therefore, are putative inhibitors of the efflux pump or pumps of *E. coli*, the inhibition of efflux pumps by these agents may result in larger amounts of compound reaching plasmid DNA targets during the division of the bacterium. Because the compounds at the concentrations used do not inhibit the replication of the bacterium, inhibition of plasmid replication subsequent to the division of the daughter *E. coli* cells means that whereas one of the daughters contains a plasmid, the other will not. If the inhibition of plasmid DNA replication is irreversible, the presence of the plasmid in subsequent generations will decrease with each replication of the bacterium. If we assume that replication of bacterium DNA is not inhibited by the agent due to its largesse in comparison to that of the vastly smaller sensitive plasmid DNA, then one would expect that the much larger F'lac K12LE140 plasmid would be less sensitive to the agent than that of the pBR322. This may explain why the SILA compounds were far more effective on the elimination of pBR322 as opposed to the elimination of the F'lac K12LE140 plasmid from the same bacterial strain. Although we tend to favour the aforementioned hypothesis, there is the possibility that elimination of plasmids may also involve their physical removal via openings created in the cell envelope. If this is true, then the largesse of the plasmid will result in less loss whereas plasmids which are much smaller can readily exit from the bacterium. However, because SILA compounds do not inhibit the replication of the bacterium at the concentrations employed in this study, there is little chance that the exit of the plasmid from the bacterium is due to the creation of openings in the cell envelope by the agent. Nevertheless, the means by which different plasmids are eliminated from the bacterial cell is not completely understood and we must therefore await the definition of that mechanism before we can ascribe the mechanisms by which SILA compounds eliminate plasmids from their bacterial host. Regardless of the mechanism, because SILA compound 421 is non-toxic, its use as an adjuvant for therapy of plasmid-mediated antibiotic-resistant *E. coli* infections of the human and the ungulate has potential.

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References

1 Schumacher H, Skibsted U, Skov R and Scheibel J: Cefuroxime resistance in *Escherichia coli*. Resistance mechanisms and prevalence. APMIS *104*: 531-538, 1996.

2 Mammeri H, Van De Loo M, Poirel L, Martinez-Martinez L and Nordmann P: Emergence of plasmid-mediated quinolone resistance in *Escherichia coli* in Europe. Antimicrob Agents Chemother *49*: 71-76, 2005.

3 Poirel L, Heritier C, Spicq C and Nordmann P: *In vivo* acquisition of high-level resistance to imipenem in *Escherichia coli*. J Clin Microbiol *42*: 3831-3833, 2004.

4 M'Zali FH, Heritage J, Gascoyne-Binzi DM, Denton M, Todd NJ and Hawkey PM: Transcontinental importation into the UK of *Escherichia coli* expressing a plasmid-mediated AmpC-type beta-lactamase exposed during an outbreak of SHV-5 extended-spectrum beta-lactamase in a Leeds hospital. J Antimicrob Chemother *40*: 823-831, 1997.

5 Vuye A, Verschraegen G and Claeys G: Plasmid-mediated beta-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* resistant to ceftazidime. Antimicrob Agents Chemother *33*: 757-761, 1989.

6 Liebana E, Batchelor M, Hopkins KL, Clifton-Hadley FA, Teale CJ, Foster A, Barker L, Threlfall EJ and Davies RH: Longitudinal farm study of extended-spectrum beta-lactamase-mediated resistance. J Clin Microbiol *44*: 1630-1634, 2006.

7 Koh CL and Kok CH: Antimicrobial resistance and conjugative R plasmids in *Escherichia coli* strains isolated from animals in Peninsular Malaysia. Southeast Asian J Trop Med Public Health *15*: 37-43, 1984.

8 Livermore DM and Woodford N: The beta-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. Trends Microbiol *14*: 413-420, 2006.

9 Bouza E and Cercenado E: Klebsiella and enterobacter: antibiotic resistance and treatment implications. Semin Respir Infect *17*: 215-230, 2002.

10 Dudley M: Bacterial resistance mechanisms to beta-lactam antibiotics: assessment of management strategies. Pharmacother *15*: 9S-14S, 1995.

11 Molnar J, Mandi Y and Kiraly J: Antibacterial effect of some phenothiazine compounds and R-factor elimination by chlorpromazine. Acta Microbiol Acad Sci Hung *23*: 45-54, 1976.

12 Molnar J, Schneider B, Mandi Y, Farkas S and Holland IB: New mechanism of plasmid curing by psychotropic drugs. Acta Microbiol Acad Sci Hung *27*: 309-315, 1980.

13 Molnar J, Mandi Y and Foldeak S: Drug-receptor interaction on plasmid elimination by phenothiazines and imipramine in *Escherichia coli*. Acta Microbiol Acad Sci Hung *29*: 17-25, 1982.

14 Molnar A, Amaral L and Molnar J: Antiplasmid effect of promethazine in mixed bacterial cultures. Int J Antimicrob Agents *22*: 217-222, 2003.

15 Spengler G, Miczak A, Hajdu E, Kawase M, Amaral L and Molnar J: Enhancement of plasmid curing by 9-aminoacridine and two phenothiazines in the presence of proton pump inhibitor 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone. Int J Antimicrob Agents *22*: 223-227, 2003.

16 Amaral L, Viveiros M and Molnar J: Antimicrobial activity of phenothiazines. In Vivo *18*: 725-731, 2004.

17 Wolfart K, Spengler G, Kawase M, Motohashi N, Molnar J, Viveiros M and Amaral L: Synergistic interaction between proton pump inhibitors and resistance modifiers: promoting effects of antibiotics and plasmid curing. In Vivo *20*: 367-372, 2006.
18 Viveiros M, Jesus A, Brito M, Leandro C, Martins M, Ordway D, Molnar AM, Molnar J and Amaral L: Inducement and reversal of tetracycline resistance in *Escherichia coli* K-12 and expression of proton gradient-dependent multidrug efflux pump genes. Antimicrob Agents Chemother *49*: 3578-3582, 2005.
19 Hegyes P, Molnar J, Mucsi I, Hever A, Szabo D, Kiesig S, Lage H, Gaal D and Nacsa J: Substituted disiloxanes: method for the production thereof and the use thereof for reversal of multidrug resistance (MDR) PCT/DE00/04110, 2000.
20 Kristiansen MM, Leandro C, Ordway D, Martins M, Viveiros M, Pacheco T, Kristiansen JE, Molnar J and Amaral L: Thioridazine reduces resistance of methicillin-resistant *Staphylococcus aureus* by inhibiting a reserpine sensitive efflux pump. In Vivo *20*: 361-366, 2006.
21 Chiu IM and Lehtoma K: Direct cloning of cDNA inserts from lambda gt11 phage DNA into a plasmid vector by a novel and simple method. Genet Anal Tech Appl *7*: 18-23, 1990.
22 Ordway D, Viveiros M, Leandro C, Arroz MJ and Amaral L: Intracellular activity of clinical concentrations of phenothiazines including thioridiazine against phagocytosed *Staphylococcus aureus*. Int J Antimicrob Agents *20*: 34-43, 2002.
23 Zalatnai A and Molnar J: Effect of SILA-409, a new organosilicon multi-drug resistance modifier, on human pancreatic cancer xenografts. In Vivo *20*: 137-140, 2006.
24 Kawase M, Motohashi N, Sakagami H, Kanamoto T, Nakashima H, Ferenczy L, Wolfard K, Miskolci C and Molnar J: Antimicrobial activity of trifluoromethyl ketones and their synergism with promethazine. Int J Antimicrob Agents *18*: 161-165, 2001.
25 Martins M, Bleiss W, Marko A, Ordway D, Viveiros M, Leandro C, Pacheco T, Molnar J, Kristiansen JE and Amaral L: Clinical concentrations of thioridazine enhance the killing of intracellular methicillin-resistant *Staphylococcus aureus*: an *in vivo*, *ex vivo* and electron microscopy study. In Vivo *18*: 787-794, 2004.
26 Amaral L, Kristiansen JE, Frolund Thomsen V and Markovich B: The effects of chlorpromazine on the outer cell wall of *Salmonella typhimurium* in ensuring resistance to the drug. Int J Antimicrob Agents *14*: 225-229, 2000.
27 Molnar J, Fischer J and Nakamura MJ: Mechanism of chlorpromazine binding by gram-positive and gram-negative bacteria. Antonie Van Leeuwenhoek *62*: 309-314, 1992.
28 Amaral L and Lorian V: Effects of chlorpromazine on the cell envelope proteins of *Escherichia coli*. Antimicrob Agents Chemother *35*: 1923-1924, 1991.
29 Martins A, Couto I, Martins M, Viveiros M and Amaral L: Prolonged exposure of MRSA COL to increasing concentrations of oxacillin result in an mdr phenotype. Int J Antimicrobial Agents *29*: 302-305, 2007.
30 Spengler G, Molnár AM, Schelz Z, Amaral L, Sharples D and Molnár J: The mechanism of plasmid curing in bacteria. Curr Drug Targets *7*: 823-841, 2006.

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