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Synergistic Interaction Between Proton Pump Inhibitors and Resistance Modifiers: Promoting Effects of Antibiotics and Plasmid Curing

KRISTINA WOLFART[1], GABRIELLA SPENGLER[1], MASAMI KAWASE[2], NOBORU MOTOHASHI[3], JOSEPH MOLNÁR[1], MIGUEL VIVEIROS[4] and LEONARD AMARAL[4]

[1]*Department of Medical Microbiology, University of Szeged, 6720 Szeged, Dóm tér10, Hungary;*
[2]*Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama;*
[3]*Meiji Pharmaceutical University, Kiyose, Tokyo, Japan;*
[4]*Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, UPMM, Universidade Nova de Lisboa, Rua da Junqueira 96, 1349-008 Lisboa, Portugal*

Abstract. *A proton pump-deleted mutant E. coli, AG100 A, had greater sensitivity to ampicillin, tetracycline and erythromycin than the wild-type parent E. coli AG100 containing the proton pump. This antibiotic sensitivity was further increased by resistance modifiers such as the Ca^{2+} channel blocker (±) verapamil (VP) and the calmodulin antagonist promethazine (PMZ). Whereas the newly-synthesized trifluoromethyl-ketone (TF) enhanced the activity of these antibiotics against the wild-type strain, it did not enhance the activity of ampicillin against the proton pump-deleted mutant. These results suggested that TF14 had an inhibitory effect on the proton pump. Elimination of plasmids from another strain of E. coli, K12, was promoted by PMZ and 9-amino-acridine (9-AA), but not by TF14 alone. However, combinations of TF14 with either PMZ or 9-AA enhanced the plasmid elimination capacity of the latter compounds. The combination of TF14, PMZ and VP proved that the Ca^{2+} channel blocker was not effective by itself. These results collectively suggest that TF14 inhibited the proton pump of E. coli and that it was this pump which, when inhibited by TF14, allowed more PMZ to reach its plasmid elimination target.*

Bacterial resistance to antibiotics has markedly increased during the past two decades being related to the improper administration of antibiotics (1). Although resistance to antibiotics has been shown to involve many mechanisms, some of which are genetic (2) and others phenotypically determined (3-7), the two most common causes of bacterial antibiotic resistance involve the acquisition of genes from the same or different species that render an antibiotic target immune from the antibiotic (8), and the presence of an energy-dependent efflux pump (induced or acquired) that recognises the antibiotic and other unrelated compounds, quickly expelling the antibiotic before it reaches its target (9, 10). Whereas the acquisition of plasmids or other extrachromosomal material generally involves resistance to one antibiotic or its relatives, antibiotic resistance mediated by an efflux pump usually promotes resistance to two or more unrelated antibiotics (11-14). Our previous studies, as well as those of others, have shown that phenothiazines caused the elimination of plasmids from *E. coli*, inhibited the growth of the bacteria (15-17) and inhibited efflux pumps present in tumour cells (18). The question of whether each of these phenothiazine-promoted effects took place in a wild-type *E. coli* strain with an intact multidrug efflux pump system (AcrAB-TolC energised by the proton motive force), and in the mutant whose proton efflux pump has been genetically-deleted (19-21), were investigated here. The inhibition of efflux pumps by established and newly-synthesized compounds, thus bringing about the reversal of antibiotic resistance (22), may provide a viable alternative in the management of antibiotic-resistant infections.

Correspondence to: Leonard Amaral, Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 96, 1349-008, Lisboa, Portugal. Tel: 351 21 365 2653, Fax: 351 21 363 2105, e-mail: LAmaral@ihmt.unl.pt

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Materials and Methods

Bacterial strains. Escherichia coli K12 AG100 (*argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44*), wild-type containing a fully functional acrAB efflux pump, and *Escherichia coli* K12 AG100A (*Δacr* AB), with an inactive efflux pump due to mutation, were used. These strains have been characterised by Hiroshi Nikaido (Departments of Molecular and Cell Biology and Chemistry, University of

California, Berkeley, CA, USA) (19), who kindly provided them for this study. The above *E. coli* strains were transformed with pBR322 plasmid (amp^r, tet^r) to *E. coli* AG100 pBR322 and *E. coli* AG100A pBR322. The *E. coli* K12 LE140 strain was also employed in this study.

Growth media. Minimal-tryptone-yeast extract (MTY) (23) broth supplemented with 40% glucose (EGIS Pharmaceutical Company, Budapest, Hungary) (10^{-4} mg/ml final concentration) and 0.1 M $MgSO_4$ ($2.5x10^{-5}$ M final concentration) was used for culturing the bacterial strains in liquid. Isolation of the colonies was performed on MTY agar (1.5%), supplemented with ampicillin (0.1 mg/ml final concentration) and tetracycline ($1.25x10^{-2}$ mg/ml final concentration), and used for replica plating and for culturing and selection of the *E. coli* strains transformed with the pBR322 plasmid (amp^r, tet^r).

Compounds. The following compounds were purchased from the respective companies: Pipolphen® (promethazine) (PMZ) (EGIS Pharmaceutical Co.), Verapamil® (verapamil) (VP) (Chinoin Pharmaceutical Co., Budapest, Hungary), Melipramin® (imipramine) (EGIS Pharmaceutical Co.), Penbritin® (ampicillin) (AMP) (Beecham Pharmaceutical Ltd., Budapest, Hungary), erythromycin (ER) (erythromycin lactobionat) (Chimimport, Budapest, Hungary), tetracycline (TET), 3- (4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium- bromide (MTT) (MERCK, Budapest, Hungary) and 9-amino-acridine propanone (9-AA) (Aldrich-Chemie, Steinheim, Germany). 3-(2-Benzoxazolyl)-1,1,1-trifluoro-2-propanone (TF14) was synthesized by reacting 2-methyl-benzoxazole with trifluoroacetic anhydride in the presence of pyridine (24).

Preparation of solutions for plasmid DNA extraction. All solutions were freshly prepared on the day of experiment. Solution I: 1 M Tris-HCl (pH=8) (2.5%), 0.5 M glucose (10%) and 0.25 M EDTA (4%) dissolved in distilled water. Lysozyme (Reanal, Budapest, Hungary) dissolved in solution I (5 mg/ml). Solution II: 10 N NaOH (2%) and SDS (10%) dissolved in distilled water.

Determination of minimum inhibitory concentration (MIC). The dispensing and dilution of the bacteria/reagents/compounds to a 96-well microplate was conducted with the aid of an 8 multi-barrel pipette. Specific wells and columns of the microplate were designated to receive specific concentrations of the compounds alone or in combination. Details describing the precise compound or compounds, their concentrations and sequence of addition when pertinent, are provided by the respective legends of the tables or figures of the text. All microplates were incubated at 37˚C for specific periods of time. Ten ml of the solution of MTT (5 mg/ml in phosphate-buffered saline (PBS)) were added to each well in order to evaluate the rate of bacterial growth, since the mitochondrial dehydrogenase of living cells reduces MTT resulting in a blue formazan salt. The plates were incubated for 4 hours at 37˚C and the minimum inhibitory concentration value of any compound against a given strain of *E. coli* was determined.

Checkerboard microplate method. The Microplate Checkerboard technique, formulae and the interpretation of data for the determination of the effect of two drugs in combination at distinct concentrations has been previously described (25, 26).

Transformation of bacteria with pBR322 plasmid. One ml of an overnight YTB culture of an AMP-TET-sensitive bacterial strain was added to 100 ml YTB broth and incubated at 37˚C until an optical density (OD) of 0.25-0.30 at 600 nm was reached. The culture was transferred to an ice bath for 10 minutes, centrifuged at 4500 rpm for 10 minutes, then the supernatant was removed and the pellet re-suspended in 50 ml ice-cold 0.1 M $MgSO_4$, followed by centrifugation in a cold tube at 4500 rpm for 10 minutes. The supernatant was then removed and the pellet was re-suspended in 3.3 ml ice-cold 0.1 M $CaCl_2$ and incubated for 1 hour in an ice bath. Two hundred μl of these cultures were transferred into tubes containing 1 μl of pBR322 (plasmid carrying the genes for AMP and TET resistance) and the tubes kept in an ice bath for 30 minutes, before being rapidly transferred to 42˚C for 1 minute in order for the cells to be "shocked". One ml of YTB broth was added to the "shocked" cells and the tubes incubated for 1 hour at 37˚C. These cells were then centrifuged in an Eppendorf centrifuge for a few seconds and 800 ml of supernatant removed. The cells were re-suspended in the remaining supernatant and an aliquot of 200 ml was plated onto YTB agar containing the plasmid that bestowed resistance to AMP and TET. The colonies present on these plates contained the plasmid.

Plasmid elimination method. One colony of bacteria transformed with pBR322 plasmid was added to MTY broth media (5 ml), supplemented with glucose and $MgSO_4$, and incubated for 24 hours at 37˚C. From a 10^{-4} dilution of this overnight initial culture, 2 ml were transferred to tubes containing 200 ml of MTY broth media, mixed and distributed in 5-ml aliquots into test tubes. Different concentrations of the curing compounds (compounds known to cause the elimination of plasmids) such as PMZ were added to the cells, which were then incubated for 24 hours at 37˚C. 10^4 and 10^5 dilutions of these cell suspensions were made and aliquots of 100 ml plated onto YTB agar and the plates incubated for 24 hours at 37˚C. The colonies present on these master plates were transferred by the velvet replica plating technique onto YTB agar containing AMP and TET (replica plate). The plates were further incubated at 37˚C for 24 hours, after which time the distribution of colonies present in each of the respective plates was compared by simple over-laying methods (27). Colonies present on the replica plates contained the pBR322 plasmid, coding for resistance to AMP and TET. Colonies present on the master plate but not growing on the replica plate provided evidence of plasmid elimination promoted by PMZ or another compound tested. Comparing the number of colonies on both plates provides an estimate of the percent plasmid elimination (cure index) produced by a given compound.

Plasmid DNA extraction. The extraction of plasmid from transformed strains of *E. coli* was conducted in accordance with the method previously described (28).

Results

The antibiotic sensitivity of two *E. coli* strains (*E. coli* AG100 – wild-type and *E. coli* AG100A – mutant) to various antibiotics and the interaction of the antibiotics and a known resistance modifier (19, 29), the Ca^{2+}-calmodulin antagonist PMZ, were evaluated by the agar diffusion method. According to the size and shape of the

Table I. *The MIC of antibiotics, known and newly-synthesized resistance modifiers against strains of E. coli that differed with respect to the active presence of an efflux pump.*

Compounds	MIC values (mg/L)	
	E. coli AG 100 (wild)	*E. coli* AG 100A (mutant)
Promethazine	156	78
Imipramine	160	80
Ampicillin	5	1.25
Tetracycline	6	0.40
Erythromycin	80	1.25
Verapamil	500	250
TF14	8	4

MIC = minimum inhibitory concentration.

Table II. *Elimination of the pBR322 plasmid with promethazine from E. coli AG100 (wild) and AG100A (mutant) strains.*

Promethazine (mg/L)	Colonies without plasmid (%)	
	E.coli AG 100 (wild)	*E.coli* AG 100A (mut)
0	0	0
40	0	0
60	0	0
80	0	11
100	1	MIC
120	13	
140	10	
160	MIC	

MIC = minimum inhibitory concentration.

zones of inhibition, there was no interaction between PMZ and the representative antibiotics of the aminoglycoside, macrolide and tetracycline groups (data not shown). Thus, one can conclude that PMZ did not modify the sensitivity of the wild-type and mutant strains of *E. coli* to the antibiotic tested.

The antimicrobial activites of PMZ, imipramine, antibiotics and TF14 against the wild-type and proton pump-deleted mutant *E. coli* strains were determined by the broth dilution method. As shown in Table I, the proton pump-deleted mutant strain of *E. coli* was at least twice as sensitive to these compounds as was the wild-type parental strain.

The plasmid curing ability of PMZ was evaluated using the two bacterial strains transfected with pBR322 plasmid (tet^r, amp^r). As shown in Table II, PMZ promoted the elimination of plasmids from the wild-type *E. coli* strain in a concentration-dependent manner, with the maximum curing effect taking place at a concentration just below the MIC of the compound (120 mg/L). In contrast to the wild-type strain, PMZ caused significant and maximum elimination of plasmids from the proton pump-deleted mutant at a much lower concentration (80 mg/L).

The elimination of plasmids by PMZ took place with other strains of *E. coli* and was consistent, as shown by the data obtained from four experiments conducted at different times (Table III). In as much as some compounds that have antimicrobial activity also have the ability to eliminate plasmids from *E. coli* (15), TF14 was assayed for this characteristic and found to be totally ineffective for the elimination of plasmids from *E.coli* AG100 pBR322, *E. coli* AG100A pBR322 and *E. coli* K12 LE140 (data not shown). However, as shown in Table III, TF14 in combination with PMZ, each at sub-inhibitory concentrations, enhanced the plasmid elimination effects of PMZ in a concentration-dependent manner. Acridine dyes have also been shown to

Table III. *Plasmid elimination from E. coli K12 LE140 by promethazine alone and in combination with sub-inhibitory concentrations of TF14*.*

Samples	Concentration (μg/mL)	Lac+ colonies/ plate	Lac– colonies/ plate	Plasmid curing (%)
	0	17	17	50.00
	0.05	4	60	93.75
Promethazine	0.1	27	141	83.93
60 μg/mL+TF14	0.5	1	238	99.58
	1.00	-	-	-
	2.00	-	-	-
	0	25	14	35.90
	0.05	0	222	100.00
Promethazine	0.1	20	20	50.00
60 μg/mL+TF14	0.5	230	0	0
	1.00	265	0	0
	2.00	-	-	-
	0	19	6	24.00
	0.05	18	6	25.00
Promethazine	0.1	478	584	52.11
60 μg/mL+TF14	0.5	15	486	97.00
	1.00	-	-	-
	2.00	-	-	-
	0	38	30	44.12
	0.05	48	264	84.6
Promethazine	0.1	39	86	68.8
60 μg/mL+TF14	0.5	-	-	-
	1.00	-	-	-
	2.00	-	-	-

*10^{-4} and 10^{-5} dilutions

Lac + (purple colony is demonstrative of presence of plasmid containing the Lac operon).

Lac – (white colony is demonstrative of absence of plasmid containing the Lac operon).

Table IV. *Plasmid elimination from E. coli K12 LE140 by 9-amino-acridine (9-AA) alone and in combination with sub-inhibitory concentration of TF14*.*

Samples	Concentration (μg/mL)	Lac+ colonies/ plate	Lac– colonies/ plate	Plasmid curing %
	0	13	9	40.90
	0.05	29	40	57.97
9-AA	0.1	73	34	31.78
5 μg/mL+TF14	0.5	29	86	74.78
	1.00	20	105	84.00
	2.00	-	-	-
	0	25	28	52.83
	0.05	53	68	56.20
9-AA	0.1	19	138	87.90
5 μg/mL+TF14	0.5	46	289	86.27
	1.00	4	28	87.50
	2.00	-	-	-
	0	17	36	67.92
	0.05	53	116	68.64
9-AA	0.1	4	64	94.12
5 μg/mL+TF14	0.5	30	69	69.70
	1.00	-	-	-
	2.00	-	-	-
	0	19	29	60.42
	0.05	50	27	35.10
9-AA	0.1	59	44	42.72
5 μg/mL+TF14	0.5	51	122	70.52
	1.00	28	148	84.10
	2.00	-	-	-

* Sub-inhibitory concentration of TF14 used was 2 mg/L (equivalent to 25% MIC).

Table V. *Effect of combinations of concentrations of promethazine, antibiotics and TF14 on the growth of E. coli AG100 (wild) and E.coli AG100A (mutant) strains.*

	Type of interaction	
Combination	AG100 (wild)	AG100A (mutant)
PMZ + TF14	Synergy[1]	Synergy[2]
VP + TF14	Antagonism[3]	Additive[4]
AMP + TF14	Additive[5]	Indifferent[6]
TET + TF14	Synergy[7]	Synergy[8]
ER + TF14	Synergy[9]	Synergy[10]

PMZ (promethazine), VP (verapamil), TF14(3-(2-benzoxazolyl)-1,1,1-trifluoro-2-propanone), AMP (ampicillin), TET (tetracycline), ER (erythromycin). The effects of varying concentrations of compounds in combination and their effect on the growth of each strain was determined by the checkerboard method and its criteria for interpretation, as previously described (25, 26). With the exception of the combination of VP + TF14 against the wild-type, where the concentration of TF14 required to bring about complete inhibition of growth was twice the MIC of TF14, the concentration of all other compounds in combination as shown above that had an effect on the growth of either strain of *E. coli* was significantly below their MIC. Please refer to Table I for the MIC of each compound employed above. [1]PMZ (39 μg/ml) + TF14 (1 μg/ml); [2]PMZ (19.6 μg/ml) + TF14 (1 μg/ml);[3]VP (125 μg/ml) + TF14 (15.6 μg/ml); [4]VP (125 μg/ml) + TF14 (2.0 μg/ml); [5]AMP (2.5 μg/ml) + TF14 (3.9 μg/ml); [6]AMP (1.25 μg/ml) + TF14 (3.9 μg/ml); [7]TET (2.5 μg/ml) + TF14 (2 μg/ml); [8]TET (1.5 μg/ml) + TF14 (1 μg/ml); [9]ER (2.5 μg/ml) + TF14 (2 μg/ml); [10]ER (0.2 μg/ml) + TF14 (0.5 μg/ml).

have the ability to eliminate plasmids from *E. coli* (30). Sub-inhibitory concentrations of TF14 in combination with 9-AA enhanced the plasmid elimination ability of 9-AA (Table IV).

The effects of combinations of PMZ, VP, TF14 and antibiotics on the growth of wild-type *E. coli* and its proton pump-deleted mutant were evaluated by the checkerboard method, its interpretative formulae affording the opportunity to study the interaction of two compounds, each at varying concentrations and their combined activities against a given strain of bacteria (26). As shown by the data summarised in Table V, TF14 at a concentration well below its MIC, and in combination with concentrations of either PMZ, TET or ER, each of which was also well below its MIC, had a synergistic effect on the growth of the *E. coli* wild-type and its filial proton pump-deleted mutant. In contrast to these responses, a concentration of TF14 (16 μg/ml) twice that of its MIC of 7.8 μg/ml produced an antagonistic effect on the growth of the wild-type strain when combined with VP. The combination of TF14 and VP, the latter at a concentration of 2 μg/ml which is well below its MIC, yielded an additive effect on the filial proton pump-deleted mutant. The presence of TF14 in combination with AMP yielded an additive effect on the wild-type strain, whereas this combination (*i.e.*, concentration of AMP needed to inhibit growth near or equal to the MIC of penicillin) did not affect the growth of the filial proton pump-deleted mutant.

Discussion

The antibiotic resistance of bacteria can be reduced by the elimination of plasmids (31), by inhibiting enzymes responsible for destroying the beta-lactams with the co-administration of clavulanic acid (32), or by inhibiting the membrane transporters (33). The wild-type and proton pump-deleted *E. coli* strains provided by Okusu *et al.* (19) afforded the means by which the newly-synthesized compound TF14, with respect to any potential antibacterial, antiplasmid or resistance modifier activity, could be studied independently of the proton pump of *E. coli*. In this study, the activity of antibiotics against the proton pump-deleted mutants was found to be significantly higher, thereby confirming the results of Okusu *et al.* (19). Similarly, the proton pump-deleted mutant was more sensitive

to the plasmid curing activity of sub-inhibitory concentrations of PMZ, a compound that is known to act as an inhibitor of calcium transport as well as an inhibitor of efflux pumps that are dependent on calcium transport (18, 33). The question of whether the greater sensitivity of the proton pump deleted-mutant to antibiotics and to TF14 was due to the absence of the proton pump was investigated using both strains. The results obtained with the use of sub-inhibitory concentrations of the combinations of TF14 and TET or ER showed that the presence of TF14 enhanced the activity of these two antibiotics against both the wild-type strain with an intact proton pump and the proton pump-deleted mutant. These results suggested that the proton pump serves no role in the resistance of the wild-type to either TET or ER. In contrast to these findings, TF14 enhanced the activity of AMP against the wild-type, whereas it did not alter the sensitivity of the proton pump-deleted mutant to this antibiotic. These results suggested that, with respect to resistance to AMP, the proton pump did have a role and that this role could be negated by the presence of TF14. Whereas the newly-synthesized compound TF14 was also shown to increase the antiplasmid effect of PMZ in a concentration-dependent manner, TF14 alone did not cause plasmid curing. These results suggests that, whereas TF14 enhanced the plasmid curing activity of PMZ, the mechanism by which plasmid elimination takes place did not directly involve TF14. The indirect effects of TF14 may, however, be the result of TF14 affecting the amount of PMZ reaching the site, where it has its plasmid curing activity; because TF14 appeared to affect the proton pump of the *E. coli* AG100 strain, it seems reasonable to expect that a similar action would take place in the *E. coli* K12 LE140 strain that has an intact proton pump. It is, therefore, hypothesized that the enhancement of the plasmid curing activity of PMZ by TF14 was due to the inhibition of the proton pump; a pump which is probably required to effectively extrude PMZ. The enhancement of the plasmid curing activity of 9-AA by TF14 is similarly hypothesized. The results of this study suggested that interference with the action of the proton pump can render a bacterium more susceptible to the activity of an antibiotic. Taking this concept one step further, the inhibition of the proton pump by an agent such as a TF14 in a concentration-dependent manner may allow the use of certain antibiotics at concentrations well below their toxic range (34-36). It remains for future studies to provide further evidence in support of this concept.

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