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Antibiotic Resistance Reversal of Multiple Drug Resistant *Escherichia Coli* Using Phenoxazine Derivatives

BC Channu [1], K Girish [2], and KN Thimmaiah[3*]

[1]*Postgraduate Department of Chemistry, Maharani's Science College for Women, Mysuru, Karnataka, India*
[2]*Postgraduate Department of Microbiology, Maharani's Science College for Women, Mysuru, Karnataka, India*
[3]*Department of Chemistry, Northwest Mississippi Community College / University of Mississippi, Desoto Center, Southaven, MS 38671, USA*

ABSTRACT

Five 2,10-disubstituted phenoxazines (10-3'-N-bis(hydroxyethyl)amino]propyl]phenoxazine BPP; 10-3'-N-bis(hydroxyethyl)amino]propyl]-2-chlorophenoxazine BPCP; 10-4'-(N-diethylamino)butyl]-2-chlorophenoxazine DBCP; 10-(3'-N-morpholinobutyl)-2-chlorophenoxazine MBCP; 10-(3'-N-piperidinobutyl)-2-chlorophenoxazine PBCP) were studied for their ability of potentiating antibacterial activity of seven antibiotics such as streptomycin, gentamicin, kanamycin, amikacin, spectinomycin, benzylpenicillin and amoxicillin against resistant strains of Escherichia coli, E. coli *K12 MG 1655 and* E. coli *ST 58. All the five phenoxazine derivatives exhibited significant potentiation of antibacterial activity of all the antibiotics up to 16-fold in vitro against both the two bacterial strains studied, except the two penicillins (benzylpenicillin and amoxicillin). The results of present investigations revealed that 2,10-disubstituted phenoxazines (BPP, BPCP, DBCP, MBCP and PBCP) could successfully reverse the multiple antibiotic resistance (MDR) in* E. coli *K12 MG 1655 and* E. coli *ST 58, making them sensitive to antibiotics.*

Keywords: Phenoxazine derivatives, antibiotics, multiple drug resistance, reversal, *Escherichia coli*

INTRODUCTION

Antibacterial therapy has played a very important role in the treatment of infectious diseases. However, the repeated indiscriminate use leads the antibiotics to become ineffective due to development of drug resistance by organisms [1]. In the past few decades, the rapid emergence of bacterial resistance has been observed. Bacteria have mutated or have acquired new genes producing novel machinery to overcome the action of many antibiotics [2]. In recent years, many new antibiotic-resistant strains have been isolated from patients throughout the world. Antibiotic resistance causes great therapeutic and economic burden in the treatment of infectious diseases and it may threaten the success of antimicrobial chemotherapy. It is estimated that antibiotic resistance increase the hospital stay and morbidity rate two-fold [3]. The problem of explosive escalation of antimicrobial resistance has only been worsened by a steady decrease in the number of new antibiotics introduced in the last 10–15 years [4]. The recent trends of antibiotic resistance suggest that even the newly introduced antimicrobial agents will have a short life expectancy [5]. A possible shortage of new and effective antimicrobials has pressed the need for careful and controlled use of antibiotics through the reduction of dosage per regime of treatment or by regulating prescriptions especially in animal husbandry and aquaculture [6]. However, reduced use could lead to delayed resistance development, while emergence of resistant strains is inevitable from an evolutionary view-point [7]. Therefore it has become imperative to explore alternative approaches. The discovery and development of new sources that either block or circumvent resistance mechanisms may improve the containment, treatment and eradication of these strains [8].

The design of potential reversers of bacterial drug resistance has thus become a desirable goal in the clinic. Phenoxazines are a group of nitrogen-containing heterocylic compounds and are reported to exhibit diverse biological functions such as cytotoxic, antibacterial, antiparasitic, antimalarial, antiepileptic, anticancer, antiproliferative, tranquilising, spasmolytic, antitubercular, and anthelmintic activities [9-13]. The chemistry and biology of a number of N^{10}-substituted phenoxazines synthesized originally as modulators of P-glycoprotein-mediated multidrug-resistance (MDR) have been reported [14-16]. Thimmaiah et al. [17] demonstrated that 2-chlorophenoxazines partially reversed VLB resistance in MDR colon carcinoma cell line GC_3/cl and completely reversed the 86-fold VLB resistance in the MDR1 over expressing breast carcinoma cell line BC 19/3.

Bacteria possess a wide array of drug efflux proteins, some of them sharing structural similarity to eukaryotic efflux pumps [18]. The efflux pumps, in both the cases, reduce intracellular drug concentrations and accessibility of drugs to their sites of action, finally resulting in reduced susceptibility. Most eminent multidrug transporters are P-glycoproteins (P-gps) and phenoxazines are known to be efficient P-gp inhibitors and are therefore reported to be good modulators of multidrug resistance (MDR) [10,17]. *LmrA*, the well-characterized ABC multidrug transporter of bacterial origin is classified as a member of the P-glycoprotein cluster of the ABC transporter superfamily, suggesting that P-glycoprotein type of transporter is conserved from bacteria to man [19]. Thus, it has been speculated that efflux pump inhibitors (EPIs) developed to overcome efflux in eukaryotic cells may also be used to battle bacterial resistance. Since the phenoxazines that are efficient P-gp inhibitors have been found to be very effective in circumventing drug resistance in cancer cells, it was decided to examine the effect of phenoxazines on the reversal of bacterial resistance. Hence, in the present investigations the efficacy of five 2,10-disubstituted phenoxazine modulators on the potentiation of antibacterial activity of antibiotics against two resistant strains of *Escherichia coli* was studied.

EXPERIMENTAL SECTION

Chemicals

In the present studies, 2,10-disubstituted phenoxazine derivatives such as (10-3'-N-bis(hydroxyethyl)amino]propyl]phenoxazine BPP; 10-3'-N-bis(hydroxyethyl)amino]propyl]-2-chlorophenoxazine BPCP; 10-4'-(N-diethylamino)butyl]-2-chlorophenoxazine DBCP; 10-(4'-N-morpholinobutyl)-2-chlorophenoxazine MBCP; 10-(4'-N-piperidinobutyl)-2-chlorophenoxazine PBCP were employed. The compounds were synthesized according to Channu [20]. The structural formulas and UV spectral data of the compounds are given in table 1.

Antibacterial agents

Benzylpenicillin and amoxicillin (Penicillins), streptomycin, gentamicin, kanamycin and amikacin (aminoglycocides) and spectinomycin (aminocyclitol) were employed.

Bacterial strains

In the present studies, the resistant strains of *Escherichia coli* such as *E. coli* K 12 MG 1655 obtained from Department of Biotechnology, University of Pune, India and *E. coli* ST 58 obtained from Department of Microbiology, JSS Medical College, Mysuru, India were used.

Antibacterial activity

For the determination of antibacterial activity, the standard turbidimetric method was used. Two bacterial strains (*E. coli* K12 MG 1655 and *E. coli* ST 58) were exposed to graded concentrations of seven antibiotics such as streptomycin, gentamicin, kanamycin, amikacin, spectinomycin, benzylpenicillin and amoxicillin. A range of concentrations varying from 1.41 to 181.8 µg/ml (two-fold dilution *viz.*, 1.41µg/ml, 2.84 µg/ml, 5.68 µg/ml, 11.36 µg/ml, 22.72 µg/ml, 45.45 µg/ml, 90.9 µg/ml, 181.8 µg/ml) were prepared according to Kotretsou et al. [21] and used for this purpose. The solutions were sterilized by passing through the membrane filters of pore size 0.45µm.

Bacterial cultures were grown in sterilized Luria broth medium at 37^{o}C at the speed of 120 rpm in a temperature controlled rotary shaker. Luria broth contained 1% tryptone, 0.5% yeast extract and 0.5% sodium chloride, pH 7.2. The O.D. of the bacteria from mid-log phase of growth was measured at 520nm and diluted in fresh medium so as to get on O.D of 0.004 (corresponding to $5X10^5$ cfu/ml). To each well of a microtitre plate, 20µl of antibiotic and 200µl of diluted bacterial suspension were added and incubated at 37^{o}C for 24–48h. At the end of incubation the effect of the antibiotics on the growth of the organisms was monitored by measuring the optical density at 490nm using ELISA reader. The minimum inhibitory concentration (MIC) of the antibiotics was determined in µg/ml. The MIC was defined as the minimum concentration of the compound showing complete inhibition of the microbial

species tested. The MIC for each of the antibiotics was determined by plotting optical density (O.D) as a function of concentration of antibiotics.

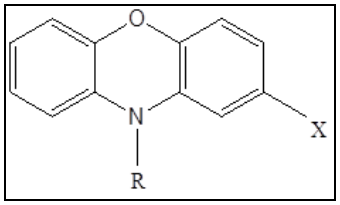

Table 1: Molecular structures and UV- spectral data of 2,10-disubstituted phenoxazine derivatives

Compound	X	R	λ_{max} (nm)	Electronic transition	Molar extinction coefficient (ε) ($lit.mol^{-1}.cm^{-1}$)	R_1 (min) (HPLC)
BPP	H	$-CH_2-CH_2-CH_2-N(CH_2CH_2OH)(CH_2CH_2OH)$	218 239 322	π →π* π →π* n →π*	48795 62385 53125	-
BPCP	Cl	$-CH_2-CH_2-CH_2-N(CH_2CH_2OH)(CH_2CH_2OH)$	244 330	π →π* n →π*	15260 8900	4.2
DBCP	Cl	$-CH_2-CH_2-CH_2-CH_2-N(CH_2CH_3)(CH_2CH_3)$	243 330	π →π* n →π*	16300 8700	7.0
MBCP	Cl	$-CH_2-CH_2-CH_2-CH_2-N$ (morpholine) O	243 330	π →π* n →π*	19000 8500	8.4
PBCP	Cl	$-CH_2-CH_2-CH_2-CH_2-N$ (piperidine) CH_2	243 330	π →π* n →π*	21400 8800	7.75

Antibacterial activity of phenoxazine derivatives against MDR E. coli strains

Stock solutions (2mg/ml) of 2,10-disubstituted phenoxazine compounds (BPP, BPCP, DBCP, MBCP and PBCP) were prepared in dimethyl sulfoxide (DMSO). Using the stock solution a range of concentrations varying from 1.41 to 181.8 µg/ml were prepared and screened against the two bacterial strains for their ability to inhibit the bacterial growth following the procedure mentioned above. From the plots of optical density versus concentration of phenoxazines, the MIC for each of the compound for both the organisms was determined.

Reversal of bacterial drug resistance by phenoxazine derivatives

Different concentrations of phenoxazines and antibiotics (less than MIC) were prepared by two-fold dilution method. To each well of a microtitre plate, 20µl of antibiotic and 20µl of phenoxazine modulator, 180µl of diluted bacterial suspension were added and incubated at 37^{o}C for 24 – 48h. At the end of incubation the effect of the antibiotics on the growth of the organisms was monitored by measuring the optical density at 490nm using ELISA reader. The MIC of the antibiotics in the presence of phenoxazine modulators was determined.

RESULTS AND DISCUSSION

Antibacterial activity of antibiotics against MDR E.coli strains

The MIC values of seven antibiotics (streptomycin, gentamicin, kanamycin, amikacin, spectinomycin, benzylpenicillin and amoxicillin) against the two bacterial strains tested (*E. coli* K12 MG 1655 and *E. coli* ST 58) are given in table 2. The efficacy of the seven antibiotics to inhibit the growth of the two organisms as shown by MIC lies in the range of 11.36 – 181.82 µg/ml. Based on the MIC values both the isolates were found to be less resistant to gentamicin. The ability of four aminoglycosides to inhibit the growth of *E. coli* K12 MG 1655 remained almost the same whereas the same antibiotics exhibited least activity against *E. coli* ST 58, suggesting the higher

resistance of latter strain to antibiotics. *E. coli* ST 58 strain appeared to be more resistant to all antibiotics tested than *E. coli* K12 MG 1655 except to benzylpenicillin.

Table 2: Minimum Inhibitory Concentration (MIC) of antibiotics against *E. coli* K12 MG 1655 and *E. coli* ST 58

Antibiotic	MIC Values (µg / ml)	
	***E. coli* K12 MG 1655**	***E. coli* ST 58**
Streptomycin	22.72	90.9
Gentamicin	11.36	45.45
Kanamycin	22.72	45.45
Amikacin	11.36	45.45
Spectinomycin	22.72	181.8
Benzylpenicillin	90.9	45.45
Amoxicillin	11.36	0

Antibacterial activity of phenoxazine derivatives against MDR *E. coli* strains

Five compounds (BPP, BPCP, DBCP, MBCP and PBCP) were screened against two bacterial strains for their ability to inhibit the bacterial growth. From the plots of optical density versus concentration of phenoxazines, the MIC for each of the compound for both the organisms were determined and the results are tabulated in table 3. Minimum Inhibitory Concentration (MIC) of all the tested 2,10-disubstituted phenoxazines against *E. coli* ST 58 was 181.8 µg / ml while against *E. coli* K12 MG 1655 it was more than 181.8 µg / ml except of DBCP which was 181.8 µg / ml.

Table3: Minimum Inhibitory Concentration (MIC) of 2,10-disubstituted phenoxazines against *E. coli* K12 MG 1655 and *E. coli* ST 58

Modulator	MIC Values (µg / ml)	
	***E. coli* K12 MG 1655**	***E. coli* ST 58**
BPP	>181.8	181.8
BPCP	>181.8	181.8
DBCP	181.8	181.8
MBCP	>181.8	181.8
PBCP	>181.8	181.8

Reversal of bacterial drug resistance by phenoxazine derivatives

Using combinatorial technique (antibiotic + modulator) MIC determinations were done with serial concentrations of antibiotics in the presence of minimum effective concentration (less than MIC) of phenoxazines. The results are also expressed in terms of fold-potentiation (i.e. ratio between the MIC of antibiotic alone and MIC of antibiotic in the presence of phenoxazine modulators), which is an index of the modulator for reversing the resistance. The effect of five 2,10-disubstituted phenoxazines (BPP, BPCP, DBCP, MBCP and PBCP) as modulators on the antibacterial activity of five antibiotics (streptomycin, gentamicin, kanamycin, amikacin, spectinomycin) is presented in table 4-8. The minimum concentration of the modulators BPP, BPCP, DBCP, MBCP and PBCP for inducing maximum activity of the antibiotics were in the range 22.72 – 90.9 µg/ml. The fold-potentiation values, calculated on the basis of MIC of antibiotics in the absence and presence of modulators were in the range of 4 to 16-fold against both the bacterial strains.

The results of effect of five modulators on the antibacterial activity of streptomycin are given in table 4. The fold-potentiation values were in the range of 4 to 16-fold in the case of both the strains. BPP and BPCP exhibited maximum modulating effect of 16-fold against both the strains. The efficacy of the five modulators on the antibacterial activity of gentamicin are shown in table 5. Modulators BPP and PBCP exhibited 8-fold potentiation against *E. coli* K12 MG 1655 while all the modulators except BPP exhibited 8-fold potentiation against *E. coli* ST 58. The results of ability of five modulators on the antibacterial activity of kanamycin are given in table 6. All the modulators except BPCP showed equal potentiating activity (8-fold) against *E. coli* ST 58 and a maximum of 4-fold potentiation by BPP in the case of *E. coli* K12 MG 1655. The antibacterial activity of amikacin in the presence of five phenoxazine modulastors is presented in the table 7. The data revealed that the amikacin's activity was enhanced by 8-fold against both the bacterial strains by BPP. In case of spectinomycin, all the five modulators exhibited maximum fold-potentiation of 16-fold against *E. coli* ST 58 while modulators BPP, BPCP and DBCP exhibited the maximum fold-potentiation of 8-fold against *E. coli* K12 MG 1655 (Table 8).

Table 4: Effect of 2,10-disubstituted phenoxazine modulators on the antibacterial activity of Streptomycin against resistant strains

Modulator	*E. coli* K12 MG 1655			*E. coli* ST 58		
	Required modulator concentration (µg / ml)	MIC of Streptomycin with modulator (µg / ml)	Fold Potentiation	Required modulator concentration (µg / ml)	MIC of Streptomycin with modulator (µg / ml)	Fold Potentiation
BPP	90.9	1.42	16	45.45	5.68	16
BPCP	90.9	1.42	16	22.72	5.68	16
DBCP	90.9	2.84	8	22.72	11.36	8
MBCP	45.45	5.68	4	22.72	11.36	8
PBCP	45.45	5.68	4	22.72	11,36	8

Table 5: Effect of 2,10-disubstituted phenoxazine modulators on the antibacterial activity of Gentamicin against resistant strains.

Modulator	*E. coli* K12 MG 1655			*E. coli* ST 58		
	Required modulator concentration (µg / ml)	MIC of Gentamicin with modulator (µg / ml)	Fold Potentiation	Required modulator concentration (µg / ml)	MIC of Gentamicin with modulator (µg / ml)	Fold Potentiation
BPP	90.9	1.42	8	22.72	11.36	4
BPCP	90.9	2.84	4	22.72	5.68	8
DBCP	90.9	2.84	4	22.72	5.68	8
MBCP	45.45	5.68	2	22.72	5.68	8
PBCP	45.45	1.42	8	22.72	5.68	8

Table 6: Effect of 2,10-disubstituted phenoxazine modulators on the antibacterial activity of Kanamycin against resistant strains

Modulator	*E. coli* K12 MG 1655			*E. coli* ST 58		
	Required modulator concentration (µg / ml)	MIC of Kanamycin with modulator (µg / ml)	Fold Potentiation	Required modulator concentration (µg / ml)	MIC of Kanamycin with modulator (µg / ml)	Fold Potentiation
BPP	45.45	5.68	4	22.72	5.68	8
BPCP	45.45	11.36	2	22.72	11.36	4
DBCP	45.45	11.36	2	22.72	5.68	8
MBCP	45.45	11.36	2	22.72	5.68	8
PBCP	45.45	11.36	2	22.72	5.68	8

Table 7: Effect of 2,10-disubstituted phenoxazine modulators on the antibacterial activity of Amikacin against resistant strains

Modulator	*E. coli* K12 MG 1655			*E. coli* ST 58		
	Required modulator concentration (µg / ml)	MIC of Amikacin with modulator (µg / ml)	Fold Potentiation	Required modulator concentration (µg / ml)	MIC of Amikacin with modulator (µg / ml)	Fold Potentiation
BPP	90.9	1.42	8	22.72	5.68	8
BPCP	45.45	2.84	4	22.72	11.36	4
DBCP	90.9	2.84	4	22.72	22.72	2
MBCP	90.9	2.84	4	22.72	11.36	4
PBCP	45.45	5.68	2	22.72	11.36	4

The modulating effect of phenoxazine derivatives on antibacterial activity of penicillins (benzylpenicillin and amoxicillin) against the two *E. coli* strains was insignificant. All the five modulators were found to possess no enhancing ability on the antibacterial activity of both penicillins tested.

Table 8: Effect of 2,10-disubstituted phenoxazine modulators on the antibacterial activity of Spectinomycin against resistant strains

Modulator	*E. coli* K12 MG 1655			*E. coli* ST 58		
	Required modulator concentration (µg / ml)	MIC of Spectinomycin with modulator (µg / ml)	Fold Potentiation	Required modulator concentration (µg / ml)	MIC of Spectinomycin with modulator (µg / ml)	Fold Potentiation
BPP	90.9	2.84	8	45.45	11.36	16
BPCP	90.9	2.84	8	22.72	11.36	16
DBCP	90.9	2.84	8	22.72	11.36	16
MBCP	90.9	22.72	1	22.72	11.36	16
PBCP	90.9	22.72	1	22.72	11.36	16

Five 2,10-disubstituted phenoxazines (BPP, BPCP, DBCP, MBCP and PBCP) have been studied for reversal of drug resistance in two of the *E.coli* strains against seven antibiotics and found that the antibiotic potentiation can occur dramatically (up 16-fold) *in vitro*. It has been demonstrated that phenoxazines seem to be working as anti-MDR agents through P-glycoprotein in MDR cancer cells [17]. Since P-glycoprotein is strongly implicated in the area of MDR in cancer chemotherapy as well as malarial resistance [22], it is speculated on similar lines, that P-glycoprotein could be the most probable candidate for bacterial resistance. It is now clear that P-glycoprotein like mechanisms are not restricted to mammalian cancer but are more common than realized throughout biota [23,24]. Two inhibitors of mamalian P-glycoprotein, reserpine and verapamil significantly increased the sensitivity of wild type bacteria (IR4) to toxic compounds. Moreover, they completely reversed the MDR phenotype of IR4 bacteria just as they do with mamalian MDR cells [25]. Phenoxazine derivatives were reported to cure plasmids in plasmid-carrying strains of *E. coli* and reduce plasmid-coded antibiotic resistance in *E. coli*. The potency of phenoxazines to sensitize the resistant organisms followed the order butyl > propyl > acetyl derivatives [26]. This plasmid curing activity of phenoxazine derivatives might also be reason for the reversal of drug resistance observed in the present studies. Though the phenoxazine derivatives were capable of modulating antibacterial activity of aminoglycocides, in contrast, were totally inactive in potentiating the activity of penicillins against both the strains of *E. coli* and the authors are unable to offer any explanation in this regard. More experiments are underway to understand the mechanism of action of phenoxazines as modulators.

The present piece of work may prove to be beneficial for searching novel potential agents against multiple drug resistant bacterial strains and reversal of their membrane protein-mediated as well as plasmid-mediated resistance. The choice of drugs depends on toxicology and yet the promise of a battery of compounds not confounded by toxicity is real and reliable, making this area a very important for the drug industry.

CONCLUSION

It can be concluded from the present results that 2,10-disubstituted phenoxazines (BPP, BPCP, DBCP, MBCP and PBCP) successfully reversed the multiple antibiotic resistance (MDR) in *E. Coli* strains (*E. coli* K12 MG 1655 and *E. coli* ST 58) mainly against aminoglycoside antibiotics making them sensitive to antibiotics. This antibiotic resistance reversal may be attributed to the inhibition of P-glycoprotein (efflux pump inhibitors) or curing of R-plasmids harbored by these MDR bacterial strains.

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