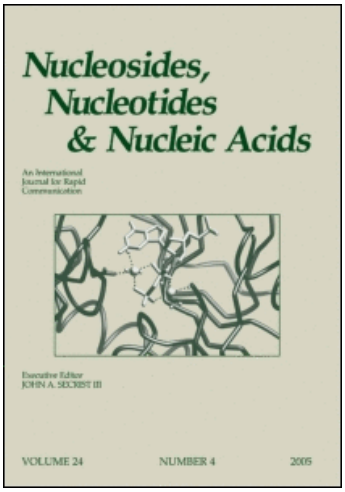

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Plasmid Loss in Plasmid-carrying Strains of Escherichia coli Treated with Phenoxazines and an Approach to Study Their DNA Binding Properties

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PLASMID LOSS IN PLASMID-CARRYING STRAINS OF *ESCHERICHIA COLI* TREATED WITH PHENOXAZINES AND AN APPROACH TO STUDY THEIR DNA BINDING PROPERTIES

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□ *The effect of subinhibitory concentrations of 2-trifluoromethyl-N^{10}-substituted phenoxazines on plasmid-coded antibiotic resistance in* Escherichia coli *was investigated. Phenoxazine treatment resulted in the loss of resistance markers to an extent of 8–63% in all the strains tested, and the disappearance of plasmid DNA in phenoxazine sensitive colonies was evidenced by agarose gel electrophoresis. The resistant strains were sensitized in the presence of phenoxazines with a concomitant reduction in the MIC (minimum inhibitory concentration) values. The UV, fluorescence spectral, and ethidium bromide displacement agarose gel assay methods revealed that phenoxazines are intercalated with plasmid DNA. Progressive addition of DNA led to a significant reduction in the peak intensity of the absorption maximum of phenoxazine derivative. Further, destabilization of ethidium bromide–DNA complex as seen from fluorescence microscopy in the presence of phenoxazines was observed. The potency of phenoxazines to sensitize the resistant organisms follows the order butyl > propyl > acetyl derivatives.*

Keywords Ethidium bromide; Fluorescence; Phenoxazine; Plasmid DNA

Plasmids in bacterial cells carry genes that govern resistance to antibiotics.[1,2] Plasmid-mediated resistance to various antimicrobial drugs has been demonstrated by different workers[3,4] based on plasmid curing experiments. Elimination of plasmid from *E. coli* by novobiocin[5]

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and coumermycin,[6] and from *S. aureus* by ascorbic acid[7,8] and ethidium bromide[9] has been reported. Hemolysin transporter protein encoding plasmids were eliminated from *E. coli* by promethazine.[10] Compounds of pharmacological interest have been found among phenoxazine derivatives.[11–13] Phenoxazine derivatives enhanced tumor necrosis factor-related apoptosis.[14] Phenoxazine compounds produced by the reactions with bovine hemoglobin showed antimicrobial activity against nontuberculosis bacteria.[15] A novel phenoxazine derivative suppressed surface IgM expression in DT 40B cell line.[16] Recent reports have shown N^{10}-substituted phenoxazines as potent and specific inhibitors of Akt signaling in cancer cells.[17] Substitution of −H by an alkyl group on the phenoxazine ring at position-N^{10} increased the antiproliferative[18,19] and anti-MDR (multidrug resistance) activities of phenoxazines in cancer cells.[20,21] These results led us to investigate whether 2-trifluoromethyl-N^{10}-substituted phenoxazines exhibit plasmid curing activities, which makes resistant bacteria susceptible to phenoxazines. Further, to gain more insight into the nature of interaction of phenoxazines with plasmid DNA leading to plasmid loss, ligand binding measurements by absorptiometric titration, fluorescence quenching and agarose gel assay methods were performed.

MATERIALS AND METHODS

All the chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. Resistance bacterial strains used in this study were obtained from Institute of Microbial Technology, Chandigarh, India. *E. coli JM 101* was obtained from Central Food Technological Research Institute, Mysore, India. Antibiotics (kanamycin, streptomycin, penicillin-G, spectinomycin, gentamycin, and ampicillin) were obtained from Sigma Chemical USA. Agarose was obtained from Himedia Laboratories, India. Liquid cultures were grown on Luria broth (LB) at pH 7.3. 2-trifluoro-N^{10}-substituted phenoxazines were synthesized and characterized following the established methods.[22,23] Solutions of phenoxazines were prepared in DMSO. Antibiotics were dissolved (stock: 20 mg/mL) in doubly distilled water and filtered through a 0.44-mm filter. Plasmid DNA was isolated by alkaline lysis method, separated by electrophoresis on agarose gel, and stained with ethidium bromide as described by Sambrook et al.[24]

Evaluation of Phenoxazines for Their Antibacterial Activity Against Resistance Bacteria

Minimum inhibitory concentrations (MIC) of phenoxazines against various bacteria were determined by microdilution method. Broth cultures

containing approximately 1.0×10^8 CFU (colony-forming units)/mL of *E. coli* was diluted with fresh medium to get an OD of 0.004. This corresponds to 5×10^5 CFU/mL. 200 μL of this suspension was inoculated in to 96-well microplates. Then, 20 μL of serially diluted phenoxazines (1.60–200 μg/mL) were added, followed by 18 hours of incubation at 37°C. MIC values for phenoxazine compounds were determined as the lowest concentration showing a significant growth inhibitory effect against each strain tested.

Sensitization of Resistance Bacteria to Phenoxazines via Plasmid Loss

An inoculum (10^6 CFU/mL) of each strain was grown in the presence of subinhibitory concentration (25 μM) of phenoxazines followed by 18 hours of incubation at 37°C. These cultures were diluted with saline and spread on selective agar plates. The number of colonies that continues to be resistant to standard antibiotics and those that become susceptible to phenoxazines were counted. Then 20–30 susceptible colonies from each strain were selected and plasmid DNA was isolated and analyzed by agarose gel electrophoresis.

Binding Study of Phenoxazines with DNA by UV Method

To a solution containing a known amount of phenoxazine derivative, solution of the plasmid DNA was added in progressively increasing amounts till the saturation in hypochromism was observed. Determination of intrinsic binding constant (K) for a given phenoxazine–DNA complex was made using the half-reciprocal plot method.[25] The intrinsic binding constant (K) was obtained form the plot of $D/\Delta\varepsilon_{app}$ versus D according to

$$\frac{D}{\Delta\varepsilon_{app}} = \frac{D}{\Delta\varepsilon} + \frac{1}{\Delta\varepsilon K}$$

where D is the concentration of DNA in base molarity, $\Delta\varepsilon_{app} = |\varepsilon_a - \varepsilon_f|$ and $\Delta\varepsilon = |\varepsilon_b - \varepsilon_f|$, where, ε_b and ε_f are respective extinction coefficients of the complex in the presence and absence of DNA. The apparent extinction coefficient, ε_a, was obtained by calculating A_{obs}/[phenoxazine derivative]. The data were fitted to the equation, with a slope equal to $1/\Delta\varepsilon$ and a y intercept equal to $1/\Delta\varepsilon K$. The intrinsic binding constant (K) was determined from the ratio of the slope to the y intercept.

Effect of Phenoxazines on the Fluorescence Intensity of Ethidium Bromide–DNA Complex

The effect of addition of varying concentrations of five phenoxazines on the emission spectra of intercalative bound ethidium bromide–DNA complex was studied. Progressively increasing amounts of different phenoxazines were added separately into solutions containing ethidium bromide–DNA complex in Tris–HCl buffer, pH 7.4. After each addition, the solutions were mixed carefully and recorded the corresponding emission spectrum. The changes in the fluorescence intensity of the ethidium bromide–DNA complex were plotted against the concentration of each of the phenoxazines.

Destabilization of Ethidium Bromide–DNA Complex by Phenoxazines

The phenoxazine–DNA association complexes were prepared by mixing different amounts of phenoxazines with the same concentration of plasmid DNA (1 μg/reaction) in Tris–HCl buffer (pH 7.4) followed by incubation at 37°C for 5 minutes in a total volume of 20 μL. Samples were loaded onto 1% agarose gel with loading dye 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water. The samples were electrophoresed under constant electric field and stained with ethidium bromide (0.5 μg/mL) for 2 hours. Bands of DNA were detected and photographed.

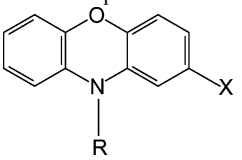
RESULTS

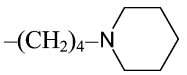
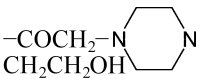
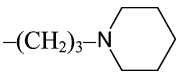
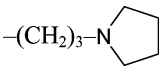
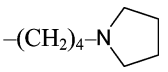
The name and structure of phenoxazine compounds used in the present study are shown in Table 1.

Determination of Minimum Inhibitory Concentration (MIC) of Phenoxazines Against Resistant Bacterial Strains

Table 2 summarizes MIC values for phenoxazine compounds (**1–5**) against four different resistant strains of *E. coli* bacteria. Out of five compounds examined, three compounds (**3**, **4**, and **5**) exerted antibacterial effect against three species of bacteria. Compounds **3** and **5** exhibited a MIC of 50 μg/mL against *E. coli HB101*, *E. coli K12*, and *E. coli 5K1592*, where as compound **4** exhibited a MIC of 50, 100, and 25 μg/mL, respectively, against the above organisms. These results demonstrate that compound **4** has the highest potency against *E. coli SK 1592* in comparison to compounds **3** and **5**. However, all four resistant bacterial strains used in this study were not affected by phenoxazines **1** and **2** (MIC $>$ 200 μg/mL).

TABLE 1 Name and structures of N^{10}-substituted phenoxazines



Comp No.	R	X	Name
1		H	10–4′-*N*-piperidinobutyl) phenoxazine (PBP)
2		H	10-[[(*β*-hydroxy-ethyl)piperazino]acetyl] phenoxazine (PiAP)
3		CF_3	10-[3′-*N*-piperidino-propyl)-2-trifluoro-methylphenoxazine (PPFP)
4		CF_3	10-[3′-*N*-pyrrolidino-propyl)-2-trifluoro-methylphenoxazine (PyPFP)
5		CF_3	10-[4′-*N*-pyrrolidino-butyl)-2-trifluoro-methylphenoxazine (PyBFP)

Phenoxazine-induced Plasmid Curing Sensitizes the Resistant Bacteria

To determine whether plasmid curing by phenoxazines is involved in sensitizing the resistant bacteria, the resistant organisms were treated with 25 μM of phenoxazine derivative and analyzed for the disappearance of plasmid-DNA by agarose gel electrophoresis. The results shown in Table 3 and Figure 1 confirm that the loss of resistance markers upon phenoxazine treatment resulted in the sensitization of the resistant bacterial strains to phenoxazine derivatives. Compounds **1** and **2** having −H at C-2 position

TABLE 2 Minimum inhibitory concentration of phenoxazines against resistance strains of bacteria

Bacterial strain genotype				MIC of phenoxazines (μg/mL)				
Test bacteria	Strain No.	Plasmid	Antibiotic resistance	1	2	3	4	5
E. coli HB101	MTCC398	PRK2013	kn^r, gn^r	>200	>200	50	50	50
E. coli K12	MTCC1263	R702	kn^r, sm^r pn^r	>200	>200	50	100	50
E. coli SK1592	MTCC392	PRT230	kn^r, sm^r	>200	>200	50	25	50
E. coli JM101	MTCC 568	PUC18	pn^r, am^r	>200	>200	200	200	200

Note. Kn^r, kanamycin resistance; gn^r, gentamycin resistance; sm^r, streptomycin resistance; pn^r, penicillin G resistance; am^r, ampicillin resistance. The results are representative of at least three independent experiments.

TABLE 3 Loss of resistance markers after phenoxazine treatment

	% Colonies sensitive to antibiotics after phenaxazine treatment				
Test bacteria	**1**	**2**	**3**	**4**	**5**
E. coli HB101	18	19	66	60	63
E. coli K12	17	20	53	45	55
E. coli SK1592	11	11	16	18	17
E. coli JM101	8	11	31	45	51

Note. Similar results were obtained in at least three independent experiments.

caused only 8–20% plasmid loss, where as phenoxazines with alkyl group at N^{10}-position and $-CF_3$ substitution at C-2 position (**3**, **4**, and **5**) caused 16–66% plasmid loss. Phenoxazines **3**, **4**, and **5** showed the plasmid loss of 60–66% in *E. coli HB101*, 45–55% in *E. coli K12*, and 31–51% in *E. coli JM101*. A total of 20–30 susceptible colonies from each experiment and each strain were analyzed. All displayed the loss of plasmid bands in agarose electrophoresis.

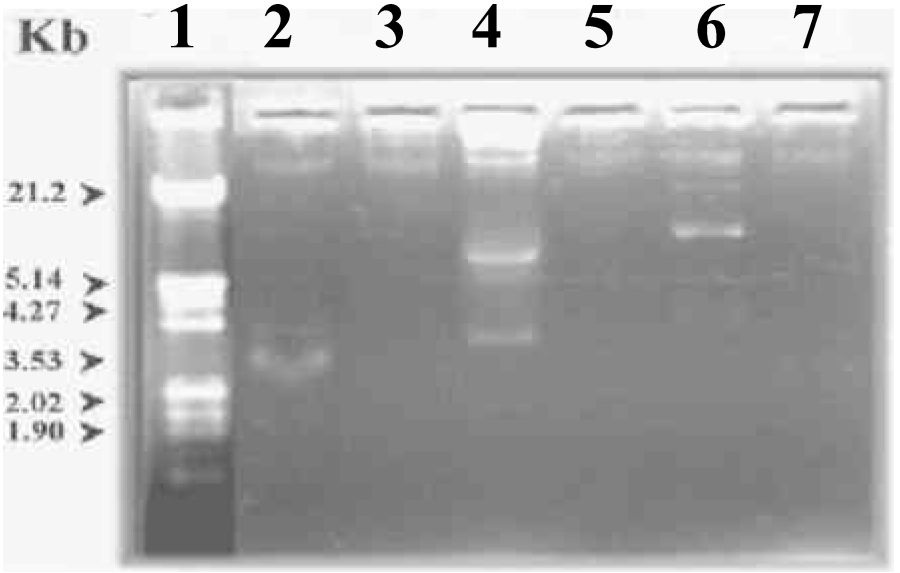


FIGURE 1 Electrophoretic screening of plasmid DNA isolated from resistant bacterial strains and phenoxazine-induced susceptible colonies. *Lane 1*: Lambda DNA *Eco*RI/*Hin*dIII double digest marker; *lane 2*: *E. coli JM 101* resistance colony; *lane 3*: *E. coli JM 101* sensitive colony; *lane 4*: *E. coli K12* resistance colony; *lane 5*: *E. coli K12* sensitive colony; *lane 6*: *E. coli SK393* resistance colony; *lane 7*: *E. coli SK393* sensitive colony.

UV-Absorptiometric Titration of Phenoxazines Against DNA

The presence of aromatic nucleobases with stacking ability and outside negative charges (phospho-diester anions of the backbone) in duplex DNA offers scope for interactive as well as electrostatic modes of binding with phenoxazines that contain planar aromatic rings. Toward this goal, five phenoxazines (**1–5**) were selected. We studied the interaction of these compounds with plasmid-DNA pUC18 by monitoring the changes in the UV-absorption spectra of the phenoxazine compounds in the wavelength range 190–250 nm upon the addition of DNA. Absorption spectra of these phenoxazines in the absence or presence of varying amounts of DNA are shown in Figure 2. All phenoxazines exhibited a strong absorption peak at λ_{max} in the range 208–214 nm. The binding of phenoxazines to DNA led to a strong decrease in the absorbance along with a strong hypochromism in the spectra of the compounds. Further, it was observed that spectra obtained upon complex formation between phenoxazines and DNA exhibit clear isosbestic points at wavelengths in the range 215–230 nm. The appearance of isosbestic point in each of these titrations suggests that there is a chemical equilibrium between the bound and free ligand with no spectrophotometrically detectable intermediate states. The peaks at 208–214 nm represent the absorption due to free ligand (phenoxazine) in solution (Trace 1 in Figure 2), while corresponding peaks represent the red shifted intercalation complex between ligand and DNA. Compounds **3** and **5** caused red shift of 4, 6, and 2 nm, respectively. The extent of hypochromicity, plotted reciprocally as A_o/A versus [DNA], was found to provide good measure of relative binding affinity (inset in Figure 2). Since hypochromism is a manifestation of stacking interactions, it is probable that there is at least partial stacking with all the phenoxazines studied here that show substantial hypochromism. In contrast, compounds **1** and **2** failed to promote hypochromism to a significant extent, which could be due to lack of binding ability of phenoxazine derivatives with DNA.

Effect of Phenoxazines on the Stability of DNA–Ethidium Bromide Complex

The effect of phenoxazines on the stability of ethidium bromide–DNA complex was examined by adding progressively increasing amounts of phenoxazine derivative to solutions containing DNA-bound ethidium bromide complex followed by monitoring the changes in the fluorescence emission spectra of the resulting mixture after each addition. Figure 3 shows the changes in the fluorescence emission spectra upon increasing amounts of phenoxazines (**1**, **3**, **5**) to DNA-bound ethidium bromide. Trace 1 represents the spectrum due to ethidium bromide alone (2.2×10^{-6} M) in Tris–HCl (pH 7.4) buffer, and addition of DNA to this solution led to

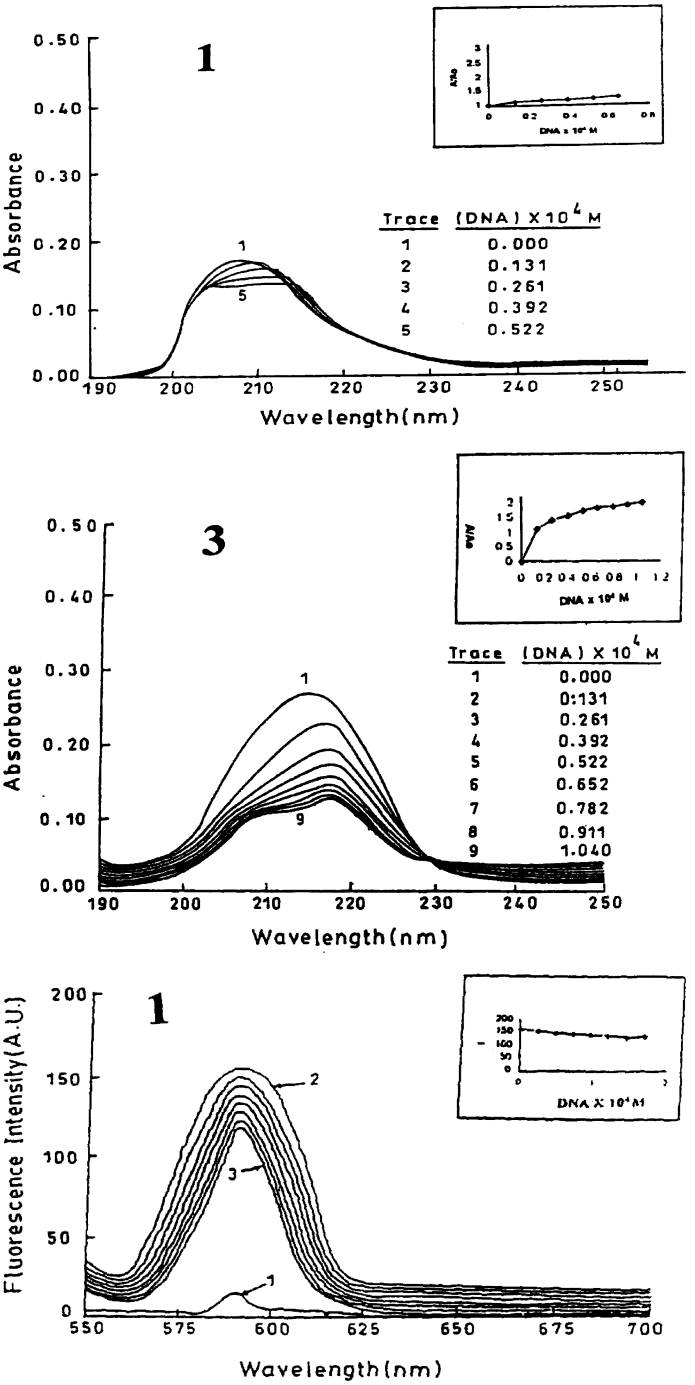


FIGURE 2 Absorptiometric titration of compound (**1**, **3**, and **5**) with DNA. *Trace 1* in all the panels shows the UV spectra due to the phenoxazine derivative alone. Subsequent traces were obtained upon incremental addition of CT DNA as shown. Insets show the plots of the relative optical density (A_o/A) versus [DNA].

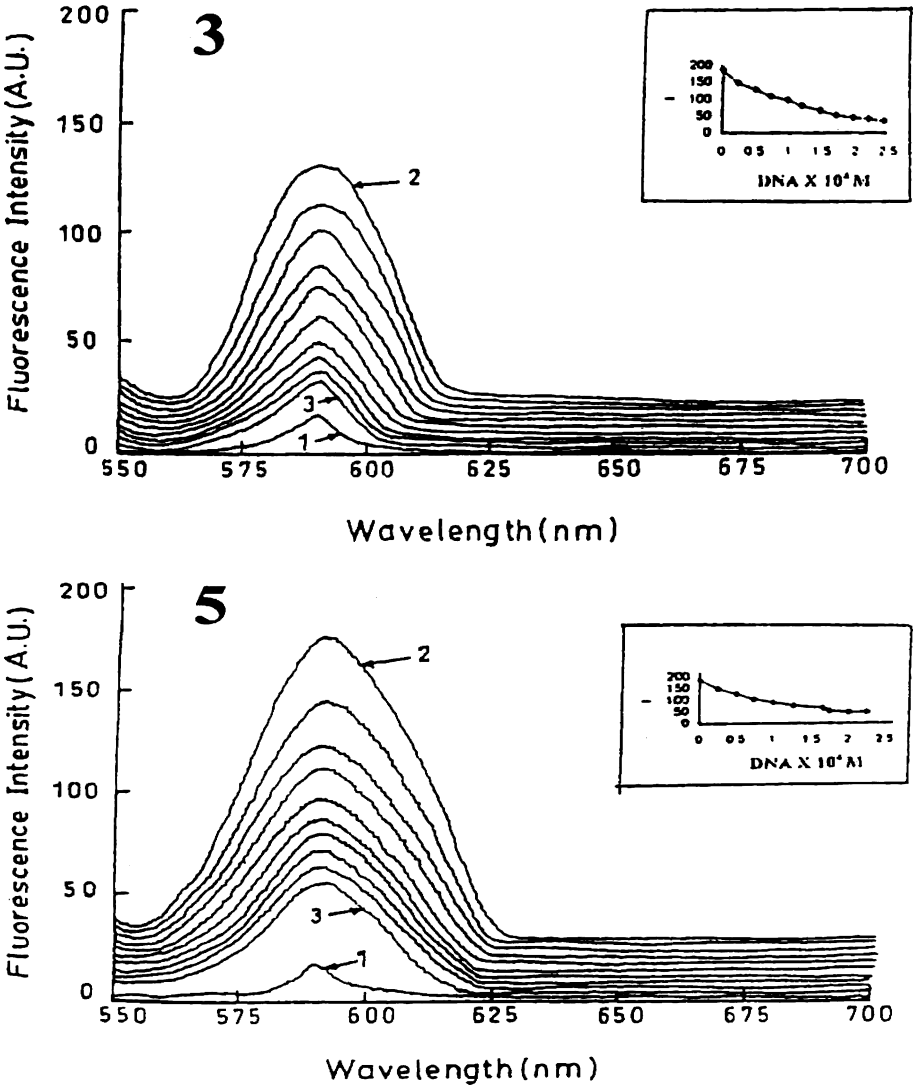


FIGURE 3 Effect of compounds **1**, **3**, and **5** on DNA- bound ethidium bromide. *Trace 1*: the fluorescence emission spectra due to 1×10^6 M ethidium bromide. *Trace 2*: the absorption spectra of fully plasmid DNA-bound ethidium bromide. *Trace 3*: the final spectra (after the attainment of saturation in fluorescence quenching obtained upon addition of phenoxazines) of a saturating concentration of a phenaxazines into the plasmid DNA–ethidium bromide complex. The corresponding insets were obtained by plotting the fluorescence intensity of (*I*) against phenoxazine concentration.

enhancement of the fluorescence emission intensity of the resulting mixture (Trace 2). The progressive addition of phenoxazines to this DNA–ethidium bromide complex led to gradual fluorescence quenching, finally reaching saturation. Trace 3 represents the maximally quenched fluorescence spectrum obtained due to addition of phenoxazines into DNA-bound ethidium bromide solution. However, addition of acetyl phenoxazine derivative (**2**)

to the DNA–ethidium bromide complex did not alter significantly the fluorescence spectra of the DNA-bound ethidium bromide complex. This is in contrast to what was observed in the experiments involving addition of phenoxazines (**3**, **4**, and **5**) containing a -propyl or -butyl group at the N^{10}-position (Figure 3). The changes in the fluorescence emission intensity of the DNA-bound ethidium bromide complex were plotted against the concentration of phenoxazines (shown as insets in Figure 3) and the resulting plots gave the relative estimates of the efficiencies of different phenoxazines in inducing destabilization of the ethidium bromide–DNA complex.

The apparent quenching of fluorescence intensity by phenoxazines could be due to a gradual release of the free ethidium bromide from the ethidium bromide–DNA complex. This is presumably due to phenoxazine-induced perturbation of DNA organization leading to dissociation of the ethidium bromide from the ethidium bromide–DNA complex. Although the efficiency in causing the destabilization of the ethidium bromide–DNA complex is varied, phenoxazines (**1**, **3**, and **5**) were able to decrease the stability of ethidium bromide–DNA complex. However, the addition of acetyl phenoxazine failed to affect the stability of DNA-bound ethidium bromide complex suggesting that there was a lack of interaction between acetyl phenoxazine derivative (**2**) and the supercoiled DNA.

Ethidium Bromide Displacement Assay

Ethidium bromide displacement assay has been used by a number of researchers to estimate the DNA binding efficiency of different classes of DNA binding molecules.[26] Since ethidium bromide is a well-characterized intercalator of DNA, the intercalation between plasmid DNA and phenoxazines can be demonstrated by agarose gel assay.

Plasmids treated with a high concentration (10^{-3} M) of acetyl-substituted phenoxazine (**2**) were visible on ethidium bromide-stained agarose gels, while those plasmids treated with equal concentration (10^{-3} M) of -propyl or -butyl derivatives of phenoxazine derivatives with $-CF_3$ group at the C-2 position were not visible. These results indicated that the N^{10}-propyl or N^{10}-butyl phenoxazines effectively displaced ethidium bromide from the plasmid. The faint visibility of the DNA bands on the agarose gel in the presence of larger concentration of phenoxazines having a -propyl or -butyl group (**3**, **4**, and **5**), even after long period of ethidium bromide staining, suggested that plasmid DNA–phenoxazine complexes are more stable than those of DNA–ethidium complexes (Figure 4). One possible explanation could be the formation of compaction between a DNA–phenoxazine molecule and concomitant alteration in the native DNA structure in water. Although the DNA conformation under this situation is changed to a compact structure, this form of DNA can still retain the

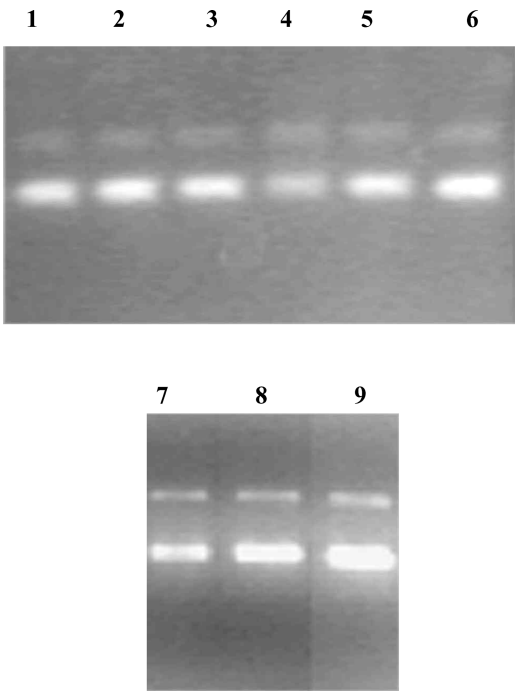


FIGURE 4 Effect of phenoxazines on the displacement of ethidium bromide from the plasmid DNA–ethidium bromide complex. *Lanes 1, 2, and 3*: plasmid DNA in the presence of 1×10^{-3} M, 1×10^{-4} M, and 1×10^{-5} M compound 1, respectively. *Lanes 4, 5, and 6*: plasmid DNA in the presence of 1×10^{-3} M, 1×10^{-4} M, and 1×10^{-5} M compound 3, respectively. *Lanes 7, 8, and 9*: plasmid DNA in the presence of 1×10^{-3} M, 1×10^{-4} M, and 1×10^{-5} M compound 5, respectively.

double-stranded organization.[27] Further, faint staining of the DNA could be also due to condensation of DNA structure resulting in insufficient space available for ethidium bromide to intercalate and stain. Such condensed DNA makes itself inaccessible to small intercalators such as ethidium bromide.

DISCUSSION

Previous studies have shown that the MDR (multidrug resistance) in antibacterial chemotherapy was correlated to the increased plasmid copy number in the resistant bacterial strains. These resistant bacteria were sensitized to phenoxazines as evidenced by their low MIC values (Table 2). The exact mechanism by which the resistant strains were sensitized by phenoxazines is unclear. It seems unlikely that this could be the result of the extracellular generation of free radicals that enter the cell and damage the DNA or other structures involved in plasmid replication or stability.

Our results suggest that *E. coli* plasmids are sensitive to the "curing" effects of phenoxazines that can be attributed to the elimination of the

coding plasmids as proved by electrophoretic screening. In case of *E. coli* JM101 and *E. coli* SK1592, curing effect is relatively less when compared to *E. coli HB101* and *E. coli K12*. This could be a consequence of high copy number of plasmids in the organisms. Hence, a possibility that phenoxazines could induce the loss of some of the copies, if not of all, resulting in sensitization. The bactericidal effect of phenoxazines on resistant strains could be a consequence of a variety of possible events:

1. The reduction in the number of plasmids within the bacterial population
2. The inhibition of the expression of the resistance determinants by interferenc with plasmid transcription or by damage to plasmid DNA
3. A synergistic effect of phenxazines, which is merely potentiation of antimicrobial activity of the drugs

Furthermore, the effect of phenoxazines differed in magnitude in different resistant strains that differed only in the resistant plasmids, Therefore, it appears that phenoxazines are acting on the plasmid and not on the host cell.

The DNA binding properties of phenoxazines clearly bring out several points of similarity and differences between the effects of phenoxazines on DNA and how the hydrophobic effects and specific charges play an important role in bringing about profound changes in DNA structure. We have examined a chromogenic probe (ethidium bromide) that interacts strongly with DNA to form an intercalated complex. The stability of the ethidium bromide–DNA complex is influenced by the addition of phenoxazines, leading to the dissociation of probe from the complex. The effect is more pronounced with phenoxazines having a -propyl or -butyl group at the N^{10}-position (compounds **3**, **4**, and **5**) as shown by UV-absorption titration, fluorescence spectroscopy, and ethidium bromide displacement assay methods. Comparison of the derivatives for their ability to bind to DNA revealed that they largely follow the order alkyl group > acetyl group chain. This trend may be due to the attachment of a polar group, $-COCH_2$ at the N^{10}-position, thereby decreasing the hydrophobicity of the molecule. In general, substitution of hydrogen by $-CF_3$ increased the ability of binding to DNA. This may be due to enhanced lipophilicity of the compounds after $-CF_3$ substitution. The efficiency of the -propyl or -butyl phenoxazines in destabilizing the probe–DNA complex is nearly three to four orders of magnitude greater than the corresponding ability of acetyl phenoxazine. Because of the presence of positive charge on nitrogen atom of the phenoxazine nucleus, electrostatic binding of these molecules to the anionic DNA phosphates is facilitated resulting in charge neutralization of the DNA backbone. This in turn reduces the inter- and intrastrand electrostatic repulsions present in native DNA phosphate backbone. As a result, under these circumstances, the DNA duplexes pack in more

compact fashion, leaving insufficient space available for the accommodation of the incoming intercalator. Thus, this results in the destabilization of probe–DNA complex. This conclusion is further supported by the lack of ethidium bromide staining of DNA bands in the presence of high concentration of phenoxazines on agarose gel electrophoresis (Figure 4). Based on these results, it is difficult to assign the exact mode of interaction, but several possibilities may exist. For example, complexation of phenaxazines with DNA might also lead to important changes in the structure of water molecules around the DNA backbone. Nevertheless, additional experiments are required to understand the exact mechanism by which phenoxazine derivatives sensitize the resistant bacterial strains.

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