Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 7, 2015

Original Article

HYDROPHOBIC INTERACTION OF 2-TRIFLUOROMETHYL-N10-SUBSTITUTED PHENOXAZINES WITH BOVINE SERUM ALBUMIN AND REVERSAL OF DRUG RESISTANCE IN BACTERIAL CELLS

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Received: 28 Apr 2015 Revised and Accepted: 30 May 2015

ABSTRACT

Objective: The objective of this study was to report the hydrophobic interaction of 2-trifluoromethyl-N10-substituted phenoxazines with bovine serum albumin and reversal of drug resistance in bacterial cells.

Methods: Binding of six compounds, 10-[3′-N-morpholinopropyl]-2-trifluoromethyl phenoxazine (1C), 10-[4′-N-morpholinobutyl]-2-trifluoromethyl phenoxazine (2C), 10-[3′-N-pyrrolidinopropyl]-2-trifluoromethyl phenoxazine (3C), 10-[4′-N-pyrrolidinobutyl]-2-trifluoromethyl phenoxazine (4C), 10-[N-piperidinoacetyl]-2-trifluoromethyl phenoxazine (5C), and 10-[N-pyrrolidinoacetyl]-2-trifluoromethyl phenoxazine (6C), to bovine serum albumin (BSA) has been measured by gel filtration and equilibrium dialysis methods. The binding of these compounds to BSA has been characterized by percentage of bound drug (β), the association constant (K), the apparent binding constant (k) and free energy (ΔF^o). The binding of phenoxazine derivatives to BSA, a serum protein that binds and transports small molecules, is correlated with their partition coefficients. Further, the ability of the phenoxazines (1C-6C) on the antibacterial activity of five antibiotics, kanamycin, spectinomycin, gentamycin, streptomycin and benzylpenicillin was examined for their ability to reverse the resistance of *E. coli K12 MG 1655* and *E. coli ST 58*.

Results: The results of displacing experiments reveal that the phenoxazine benzene rings and tertiary amines attached to the side chain of phenoxazine moiety are bound to a hydrophobic region on the albumin molecule. Among the compounds examined the butyl series seems to possess better reversing ability, suggesting that the activity could be related to lipophilicity and the extent of binding to BSA.

Conclusions: Phenoxazines are bound to albumin by hydrophobic interactions of their benzene rings. The alkyl side chain, particularly butyl chain of phenoxazines intensifies the interaction of phenoxazines with BSA. The compound that binds to a greater extent with protein possesses more activity for reversing of drug resistance.

Keywords: Hydrophobic interaction, Phenoxazines, Bacterial drug resistance, Bovine serum albumin.

INTRODUCTION

Phenoxazines comprise a large group of nitrogen-containing heterocylic compounds. They exhibit diverse biological functions such as cytotoxic, antibacterial, antiparasitic, antimalarial, antiepileptic, anticancer, antiproliferative, tranquilising, spasmolytic, antitubercular, and anthelmintic activities [1-6]. Previously, Thimmaiah *et al.* [7-13] have reported the chemistry and biology of a number of N10-substituted phenoxazines synthesized originally as modulators of P-glycoprotein-mediated multidrug-resistance (MDR). Some of these N10-substituted phenoxazines demonstrated significant anti-MDR activity by enhancing the cytotoxicity of vincristine and vinblastine against resistant cancer cells. From these results, we concluded that at least part of the activity of some of these phenoxazine MDR modulators is mediated through P-glycoprotein-dependent mechanism. As it is now established that Akt signaling protects against cellular stress, including cytotoxic agents, we have investigated whether phenoxazine derivatives inhibit Akt and induce apoptosis. The results show that a small group of novel lead phenoxazine compounds, at low micromolar concentrations specifically block Akt activation and the downstream signaling to substrates such as mTOR, p70S6 kinase, and ribosomal protein-S6. Furthermore, at low micromolar concentrations, under normal growth conditions, these small molecule inhibitors induce apoptosis in rhabdomyosarcoma cells.

Serum albumins are soluble protein constituents of the circulatory system, which has many physiological functions. The investigation of molecules and albumin binding is of importance, and has become an interesting research field in life sciences, chemistry and clinical medicine [14]. Serum albumin, the most abundant protein in blood, plays a very important role in the binding phenomenon and serves as a depot protein and transport protein for numerous endogenous and exogenous compounds. Plasma protein binding properties are primary determinants of the pharmacokinetics properties of most of the drugs, such as plasma clearance, half-life, apparent volume of distribution and the duration and intensity of pharmacologic effect. To understand the nature of drug protein interaction, the affinity of the drug for protein and number of sites are important. Bovine serum albumin (BSA), due to its structural similarity to human serum albumin (HSA) and considerable stability, has been used to replace human serum albumin in protein-drug studies [15].

Most drugs are able to bind to plasma protein when they enter into the blood plasma system of organism. Generally speaking, drugs could bind with serum albumin mostly through the formation of non-covalent complexes reversibly. Drug-protein complexes can be regarded as storage forms that alter elimination from either metabolism or clearance from plasma, thus prolonging exposure to drugs. Therefore, interaction of a drug with, and competition for, the binding sites on plasma proteins might strongly affect its distribution, elimination, as well as its pharmacodynamics and toxic properties. Binding parameters are indeed fundamental factors in determining the overall pharmacological activity of a drug, and in this context the determination of the binding parameters of drugs to albumin has become essential to understand their pharmacokinetics, pharmacodynamic, and toxicological profile. Recently, a number of investigations have been undertaken to understand the interaction between drug molecules and serum albumin [16-23].

Resistance of bacteria to many classes of antibiotics is an increasing problem worldwide. Multidrug resistance efflux pumps are recognized as an important component of resistance in both Gram-positive and Gram-negative bacteria. As a consequence of the intense fight against infections, bacteria have evolved through numerous defenses against antimicrobial agents [24], not only against drugs but also against normal xenobiotics (bile acids, etc.). Many of the protective mechanisms involve efflux transporters that effectively reduce intracellular concentrations of toxic xenobiotics, including antibacterial agents used in human and veterinary medicine. New antibacterial molecules and new therapeutic approaches are needed to overcome multidrug resistant (MDR) and extreme drug resistant (XDR) states in severe infectious diseases [25-28]. Bacteria possess a wide array of drug efflux proteins, some of them sharing structural similarity to eukaryotic efflux pumps [29]. In both cases, the transporters (efflux pumps) reduce intracellular drug concentrations and thereby impede accessibility of drugs to their sites of action, ultimately leading to reduced susceptibility. Therefore, it has been speculated that efflux pump inhibitors (EPIs) developed to overcome efflux in eukaryotic cells may also be used to battle bacterial resistance. The development of EPIs that could be used in conjunction with existing antibiotics could extend the useful lifetime of many antibiotics by improving therapeutic efficacy and by suppressing the emergence of resistant variants that might otherwise during treatment. However, clinical use of previously described bacterial EPIs, like reserpine, verapamil, gemfibrozil or cyclosporine A, was hampered by the potential for adverse effects at concentrations necessary to sufficiently inhibit the underlying transport protein. Most ATP-binding cassette (ABC) multidrug transporters are P-glycoprotein's (P-gps) and multidrug resistance-associated proteins (MRPs). Only one well-characterized ABC multidrug transporter, LmrA, is of bacterial origin. On the basis of its structural and functional characteristics, this bacterial protein is classified as a member of the P-glycoprotein cluster of the ABC transporter superfamily. LmrA can even substitute for P-glycoprotein in human lung fibroblast cells, suggesting that this type of transporter is conserved from bacteria to man. In the present article, the authors have reported the preliminary data on the ability of phenoxazines P-gp inhibitors [7-9, 12] to reverse the bacterial drug resistance.

MATERIALS AND METHODS

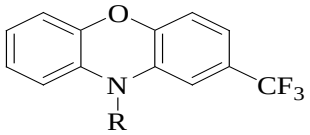
The synthesis and chemical characterization of 1C-6C has been carried out according to published method [11]. The structural formulae of the compounds are given in table 1. The compounds were purified by column chromatography and characterized by UV, IR, ^{1}H-and [13]C-NMR and mass spectral studies. The spectral data are consistent with the structures. BSA, acetyl salicylic acid and hydroxyzine were purchased from sigma (St. Louis MO). All other chemicals were of reagent grade. Resistant bacterial strains *E. coli K12 MG 1655* obtained from Department of Biotechnology, University of Pune, India, and *E. coli ST 58* obtained from the Department of Microbiology, JSS Medical College, Mysore University, Mysore, India, were used. All binding measurements were made in the presence of 0.02 M phosphate buffer, pH 6.9 containing 0.15 M NaCl and 3 m M sodium thiosulphate. Just before protein binding of a drug was determined, the pH of the sample solution was measured and where necessary adjusted to pH 6.9 with 0.1 M HCl or 0.1 M NaOH.

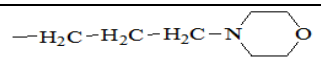
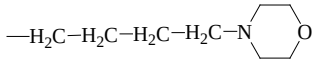
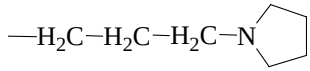
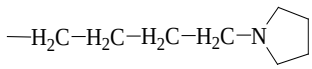
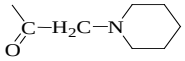
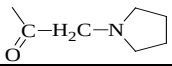
Methodologies

Separation of phenoxazine–albumin complex by gel filtration

The binding of six phenoxazine derivatives (1C-6C) to BSA was studied with the aid of gel filtration experiments. Gel filtration experiments were performed on a 20 x 1.3 cm columns of sephadex^R G-50 fine (Pharmacia) at 22 °C, equilibrated with standard buffer solution, the flow rate being maintained at 25 ml/h. 20 ml of the BSA solution (1%) containing 1.0 x 10^{-4}M phenoxazine modulator, after incubated at 37 °C for 6 h, was loaded on to the column and washed with standard buffer. The effluent from the column was collected into fractions of 3 ml. 1.5 ml of each fraction was used to determine the protein concentration and the remaining 1.5 ml for the estimation of phenoxazine modulator. The albumin content was determined by the biuret method and phenoxazine modulator estimated after extracting with n-heptane colorimetrically in 50% sulphuric acid containing 10 mg % $FeCl_3$.

Table 1: Name, molecular structure, molecular weight, $\log_{10}P$ values and pK_a of phenoxazine derivatives



Comp No.	Name	R	MW	$\log_{10}P$	pK_a
1C	10-[3^1-N-morpholinopropyl]-2-trifluoromethylphenoxazine		378	1.60	4.47 8.90
2C	10-[4^1-N-morpholinobutyl]-2-trifluoromethylphenoxazine		392	2.00	4.75 8.97
3C	10-(3^1-N-pyrrolidinopropyl)-2-trifluoromethylphenoxazines		363	1.80	4.25 8.30
4C	10-(4^1-N-pyrrolidinobutyl)-2-trifluoromethylphenoxazine		377	2.52	4.65 8.90
5C	10-(N-piperidinoacetyl)-2-trifluoromethylphenoxazine		377	1.5	4.87 9.30
6C	10-(N-pyrrolidinoacetyl)-2-trifluoromethylphenoxazine		363	1.45	5.15 8.95

Study of interaction of phenoxazines with BSA by equilibrium dialysis method

Sample solution (20 ml) containing BSA (1%) and one of the modulators (1C-6C) in the concentration range 0.1 to 5 × 10^{-4} M was taken in a 50 ml centrifuge tube and incubated at 37 °C for 6 h in a shaking water-bath incubator. For each of four dialysis tubings (3/4" diameter), 4 ml of the above reaction mixture was pipetted out and after closing, the dialysis tubing was immersed in standard buffer solution taken in a measuring jar. The dialysis tubings were agitated up and down mechanically (12 h, 22 °C). At the end of the dialysis experiment, the free phenoxazine modulator from the buffer medium was extracted with chloroform. After evaporating the organic layer, the phenoxazine concentration was measured colorimetrically using 4 ml of 50% sulphuric acid containing 10 mg % $FeCl_3$.

Displacement of phenoxazine modulator from BSA binding site by equilibrium dialysis

The displacement of phenoxazines by hydroxyzine or acetyl salicylic acid was studied by means of equilibrium dialysis. In these experiments, the binding of the phenoxazine modulator to BSA was determined after the displacing agents, hydroxyzine or acetyl salicylic acid, were added to the incubation mixture.

Measurement of lipophilicity

The relative lipophilicity at pH 7.4, of each of the compounds used in this study was assessed using an adaptation of the method of Zamora *et al.*[30]. This method involves measuring the partitioning of modulator between 1-octanol and 0.1M PBS (pH 7.4). HPLC grade 1-octanol was pre-saturated with aqueous buffer and conversely, buffered aqueous phase was pre-saturated with HPLC grade 1-octanol before use. The modulator was dissolved in aqueous phase buffer/octanol at a final concentration of 1 x 10^{-4}M, an equal volume of 1-octanol/buffer was added and the tubes were then continuously inverted for 15 min (experiments carried out over time intervals ranging from 5 to 60 min confirmed that equilibration was reached within 15 min). The final concentration of modulator in both aqueous and 1-octanol fractions was assessed by measuring the UV absorbance of these experimental fractions. The partition coefficient, P, was determined by dividing the concentration of modulator in the 1-octonal by the concentration in the aqueous phase. $\log_{10}$P was used as a measure of lipophilicity.

Cultures and other reagents for bacterial work

Bacterial cultures were grown in sterilized luria broth medium at 37 °C at a speed of 120 rpm in a rotatory shaker. Luria broth contained 1% tryptone, 0.5 % yeast extract and 0.5% sodium chloride at pH.7.2. Stock solutions of antibacterial drugs were prepared by using standard protocols and sterilized by passing through membrane filters (FH, pore size 0.5 µm and HA pore size 0.45 µm).

Determination of minimum inhibitory concentration (MIC) of standard antibiotics and

2-trifluoromethyl- N^{10}-substituted phenoxazines

Dilutions from the stock solution were made by two-fold dilution method. Twelve different concentrations of standard antibiotics solutions (gentamycin, streptomycin, spectinomycin, kanamycin and benzyl penicillin) and phenoxazine derivatives (**1C-6C**) were prepared by two-fold dilution method for the assay. The different isolates of resistant bacteria cultures (*E. coli* K12 MG 1655 and *E. coli* ST 58) were taken into Luria broth medium and incubated at 37 °C (speed 120 rpm) in a temperature controlled shaking water both. The optical density (O. D) of the bacteria from mid-log phase growth was measured at 520 nm and diluted in fresh medium so as to get an O. D of 0.004 (corresponding to ~ 5 x 10^5CFU/ml). To each well of the micro-titre plate, 20 µl of antibiotic (or phenoxazine modulator) and 200 µl of diluted bacterial suspension were added and incubated at 37 °C. The growth was usually judged at 24 and 48 h by measuring O. D using ELISA reader at 490 nm. Triplicates were maintained and the experiments were conducted twice.

Determination of minimum inhibitory concentration (MIC) of antibiotics in the presence of phenoxazine chemo sensitizers (1C-6C)

Eight different concentrations of phenoxazines (less than MIC) and 12 concentrations of antibiotics were prepared by two-fold dilution method. To each well of the micro liter plate, 20 µl of phenoxazine chemo sensitizer, 20 µl of antibiotic and 180 µl of diluted bacterial suspension were added and plates were incubated at 37 °C. The growth was judged as described above. Triplicates were maintained and the experiments were conducted twice.

RESULTS AND DISCUSSION

The drug-albumin complex may be considered as a model for gaining fundamental insights into drug-protein binding. General rules of protein binding gained from this model could apply at least partially to the drug receptor complex provided that the receptor has a protein structure. The determination of albumin binding of several structurally related compounds is a valuable tool for identifying the groups of drug molecules which are involved in binding and for characterizing the binding forces concerned with the interaction of drugs with protein.

The binding of analogous phenothiazine derivatives to BSA has been studied [23]. Although, most of the authors obtained total binding constants of the same order of magnitude, the number of binding sites varied considerably. It has been found [31] that the number of binding sites on BSA for promazine and chlorpromazine changed with the concentration of drugs, higher numbers being obtained at higher drug concentrations. The results suggested that phenothiazine derivatives are bound by hydrophobic interaction with the aromatic amino acids of the BSA molecule and that under the influence of high drug concentrations, the number of available sites increased by swelling and unfolding of the BSA molecules in solution. Glasser and Krieglstein [32] correlated the $\log_{10}$P of some phenothiazine drugs and related compounds with their log (β/α) values, 'β' and 'α' being the fractions of bound drug and free drug, respectively. A fairly good linear correlation (r = 0.969) for five 10-dimetylaminopropyl derivatives of phenothiazine was obtained. The obtained data may help us in gaining some insights on a possible drug/protein interaction. Since phenoxazines are analogous to phenothiazines structurally (oxygen in place of sulfur at position 9), similar studies have been extended to unravel the mode of interaction between phenoxazines and bovine serum albumin.

Table 2: Symbols, dimensions and methods of analysis of the parameters used

Parameter	Symbol	Dimension	Method of analysis
Total concentration of phenoxazine derivative	C	M	By weight colorimetry
Concentration of free phenoxazine derivative	C_f	M	Gel filtration, dialysis
Concentration of bound phenoxazine derivative	C_b	M	$C_b = C - C_f$
Concentration of albumin	C_a	g/100 ml	By weight. biuret method
Percentage of free phenoxazine derivative	α	%	Gel filtration, dialysis
Percentage of bound phenoxazine derivative	β	%	$\beta = 100 - \alpha$
Specific binding capacity	$\bar{r}$	M/M	$\bar{r} = C_b/C_a$ in mol
Regression coefficient	m	-	fig. 2.
Apparent binding constant	k	-	$k = C_b/C_f$ (fig. 2)
Association constant	K	10^4M^{-1}	Scatchard plot (fig. 3)
Free binding energy	ΔF °	Cal/M	$\Delta F° = -2.303RT \log_{10} K$
Partition coefficient	P	M/M	Partition between *n*-octanol and buffer, pH 7.4

Evaluation of binding parameters

The study of binding of six 2-trifluoromethyl-N^{10}-substituted phenoxazine modulators (1C-6C) to BSA by dialysis experiments was characterized by the parameters namely, percentage of bound modulator (β), the association constant (K), the apparent binding constant (k) and free energy (ΔF°) (table 3). The symbols, dimensions and methods of analysis of the values used to characterize the protein binding and hydrophobic character of the modulators are summarized in table 2. The results of gel filtration

experiments revealed that the bound modulator moves with the velocity of BSA. When experiments were performed in series for one substance, only the fractions after the protein zone have been assayed for phenoxazines.

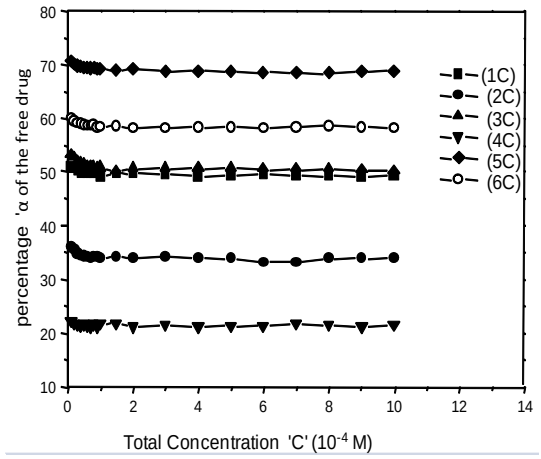


Fig. 1: Binding of varying concentration of phenoxazine modulators to bovine serum albumin. Ordinate: percentage of free phenoxazine derivative. Abscisssa: total concentration of phenoxazine derivative (10^{-4} M). Binding measurements were carried out in a 1% BSA solution (pH 6.9, 22 °C, incubated at 37 °C for 6 h). Each point represents the mean value of the two experiments

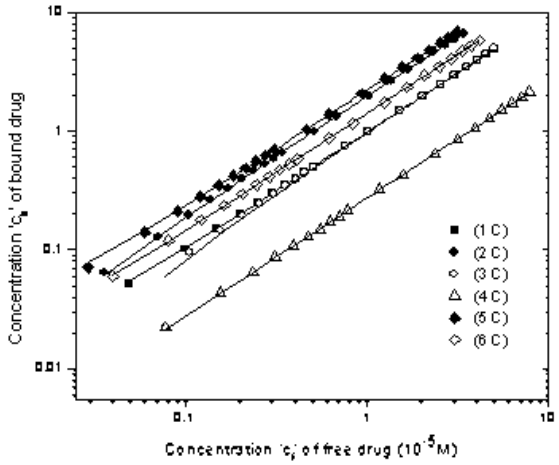


Fig. 2: Binding capacity of 1% albumin solution for phenoxazine derivatives. Ordinate: concentration "C_b"of bound phenoxazine derivative (0.01x10^{-4}M to 10x10^{-4}M). Abscissa: concentration of free phenoxazine derivative (0.1x10^{-5}M to 100x10^{-5}M). Binding measurements were carried out in a 1% BSA solution (pH 6.9, 22 °C, incubated at 37 °C for 6 h).

This plot is performed in order to obtain the regression coefficient '*m*' and apparent binding constants '*k*', see also table 2. For statistical data see table 4**. Each point represents a single experiment**

The effect of concentrations of 2-trifluoromethyl-N^{10}-substituted phenoxazines (1C-6C) on the binding to BSA was studied by dialysis experiments. In these studies, the concentration of modulator was varied in the range 0.1 to10 x 10^{-4}M keeping the BSA concentration constant (1%). The results of these experiments revealed that the binding increased with increasing concentration of phenoxazine at low modulator/protein ratios. The amount of free drug remains the same in spite of the fact that the concentration of the modulator was further increased (Fig.1), suggesting higher number of binding sites on BSA. A similar observation was made in the case of binding of phenothiazines to BSA [23]. The apparent binding constant 'k' and regression coefficient 'm' were calculated by plotting concentration of free phenoxazine modulator *versus* concentration of bound phenoxazine modulator (fig. 2). In order to calculate the total binding constant, *K*, Schatchard plot for the binding of the phenoxazine derivative to BSA was done (Fig.3). Comparison of *k* and *K* values within the compounds examined showed that the compounds bind to BSA in the order 4C>2C>3C>1C>6C>5C indicating that butyl chain attached to the phenoxazine nitrogen atom has increased the binding capacity of 2-trifluoromethyl-N^{10}-substituted phenoxazines. The extent of binding of these modulators to BSA was further supported by ΔF ° (table 3).

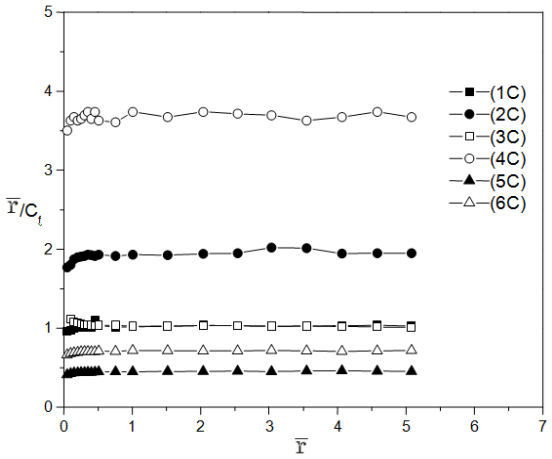


Fig. 3: Scatchard plot of binding of phenoxazine derivatives to bovine serum albumin, Ordinate: $\bar{r}/C_f$ in (10^{-4} mol^{-1}). C_f= molar concentration of free phenoxazine derivative in the albumin solution. Abscissa: $\bar{r}$ =number of moles of phenoxazine per mole of albumin. All measurements were made in a 1% albumin solution (pH=6.9, 22 °C). Each point represents the mean value of three single experiments. For total binding constant *K* see table 2. Each point represents the mean value of two single experiments

Table 3: Binding of several phenoxazine derivatives to bovine serum albumin

Phenoxazine derivative	% of bound drug (β)	Regression coefficient (m)	Apparent binding constant (k)	Association constant (K) (10^4 M^{-1})	Free binding energy ΔF° JK^{-1} mol^{-1}	$\log_{10}\beta/\alpha$
1C	48.9	0.97	0.96	0.95	22463.7	-0.0191
2C	63.9	1.97	1.77	1.75	23962.0	0.2479
3C	53.2	0.99	3.56	1.10	22823.0	0.056
4C	77.8	0.27	3.50	3.60	25731.0	0.545
5C	29.2	2.18	0.41	0.40	20315.0	-0.3846
6C	39.9	1.40	0.66	0.70	21714.7	-0.1779

β is the percentage of bound drug in a 1% BSA solution with a total concentration C=10^{-4} mol of phenoxazine modulator m, the regression coefficient and k, the apparent binding constant, was obtained from fig. 2, see also table 1, K is the association constant obtained from the scatchard plot (fig. 3), ΔF ° is the free binding energy calculated from–2.303RT Log_{10}K (R=8.314JK^{-1} mol^{-1})

Table 4: Statistical data for the binding of varying concentrations of phenoxazine derivatives with bovine serum albumin

Phenoxazine derivative	Equation of the regression line in the double logarithmic system	No. of single experiments (n)	Correlation coefficient (r)
1C	Y= 0.00762+0.97x	20	0.999
2C	Y=-0.01041+1.96689x	20	0.999
3C	Y=-0.01816+0.9865x	20	0.999
4C	Y= 0.000267+0.27x	20	0.999
5C	Y= 0.01467+2.1834x	20	0.999
6C	Y= 0.00507+1.39974x	20	0.999

Table 5: Statistical data for the displacement of phenoxazine derivatives from their albumin binding sites by acetyl salicylic acid or hydroxyzine

Compound No.+displacing agent	Equation of the regression line in the double logarithmic system	No. of single experiments (n)	Correlation coefficient (r)
1C+acetyl salicylic acid	Y=71.677+0.09042x	13	0.879
2C+acetyl salicylic acid	Y=58.8944+0.08498x	13	0.779
3C+acetyl salicylic acid	Y=69.082+0.06811x	13	0.707
4C+acetyl salicylic acid	Y=54.72+0.1089x	13	0.890
5C+acetyl salicylic acid	Y=82.03+0.0406x	13	0.316
6C+acetyl salicylic acid	Y=76.366+0.1012x	13	0.858
1C+hydroxyzine	Y=54.0108+0.025061x	13	0.706
2C+hydroxyzine	Y=47.01+0.08154x	13	0.575
3C+hydroxyzine	Y=58.18+0.04217x	13	0.525
4C+hydroxyzine	Y=42.18+0.06727x	13	0.769
5C+hydroxyzine	Y=71.3769+0.1017x	13	0.623
6C+hydroxyzine	Y=58.878+0.015726x	13	0.619

Relation between bovine serum albumin binding and hydrophobic character of phenoxazine modulators

Several authors have demonstrated a correlation between the hydrophobic character and protein binding of low molecular weight substances [33-37]. These results suggest that hydrophobic interactions play an important role in protein binding to organic compounds. However, a good correlation between protein binding and partition coefficients can be shown only for substances of structurally related groups. Hence, not only hydrophobic interactions can be present in protein binding of organic molecules, and also other binding mechanisms such as, ionic bonding, hydrogen bonding or steric effects, etc., must also be involved [38].

The hydrophobic character of six 2-trifluoromethyl-N^{10}-substituted phenoxazine derivatives (1C-6C) ($\log_{10}$ P values) was determined (table 1) to look for a possible quantitative correlation between the lipid solubility of these compounds and their protein binding ability. Analysis of the relationship between lipid solubility of modulators used in this study and the fraction, $\log_{10}(\beta/\alpha)$ where 'β' is the percentage of phenoxazine bound and 'α' is the percentage of free phenoxazine modulator showed a good correlation (fig. 4). (β/α) fraction is preferred for this type of correlation because it is directly analogous to the organic solvent-buffer partition coefficient. The following equation was arrived at from the $\log_{10}$ (β/α) and $\log_{10}$ P data for six compounds substituted with–CF_3 in position "2" of the phenoxazine nucleus.

$\log_{10}$ (β/α) =-0.834+1.624 $\log_{10}$ P (correlation coefficient, r = 0.923)

For six 2-trifluoromethyl phenoxazines, hydrophobicity decreased in the order 4C>2C>3C>1C>5C>6C. This rank order is the same as for the binding efficiency of these modulators with BSA, excepting for 5C and 6C.

Displacement of phenoxazine modulators from their bovine serum albumin binding sites

Simple aromatic substances like benzoic acid or aniline are able to displace phenothiazines from their binding sites on the albumin molecule [23]. In order to understand the BSA binding moieties of the phenoxazine modulators (1C-6C), we have studied the displacement experiments by dialysis method using hydroxyzine and acetyl salicylic acid. In these experiments, the binding of phenoxazine modulators to BSA was determined after the displacing agent, hydroxyzine or acetyl salicylic acid was added to the incubation mixture.

The participation of an alkyl chain (propyl, butyl or acetyl) of 2-trifluoromethyl phenoxazine derivatives (1C-6C) is indicated in the displacing experiments with hydroxyzine (fig. 5). Examination of the data has revealed that hydroxyzine displaces 1C by 3%, 2C by 10%, 3C by 10%, 4C by 30%, 5C by 1% and 6C by 1%. This might be explained by supposing that the aliphatic side chain between N^{10}-of 2-trifluromethyl phenoxazine nucleus and N-atom of side chain tertiary amine is not in a position to contribute to the binding of the phenoxazine modulators used.

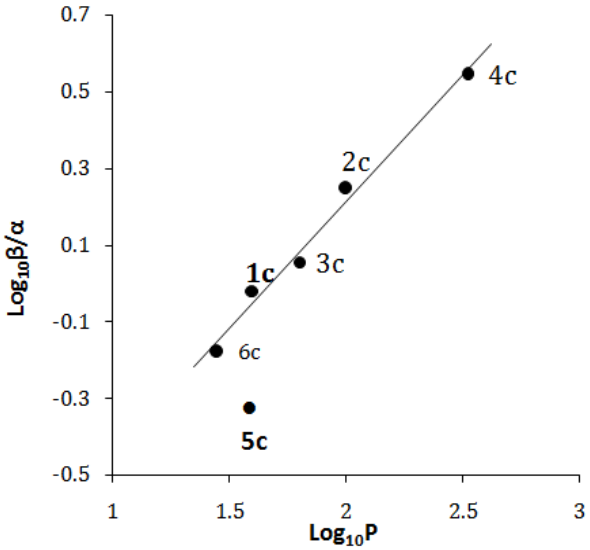


Fig. 4: Relation between albumin binding and partition coefficients of phenoxazine derivatives. Ordinate: log (β/α). *β* = percent phenoxazine derivative bound, *α* = percent free phenoxazine derivative. Abscissa: $\log_{10}$P, P=partition coefficient between n-octanol and buffer solution. The equation of the regression line: $\log_{10}\beta/\alpha$ =-0.834+1.624 $\log_{10}$P

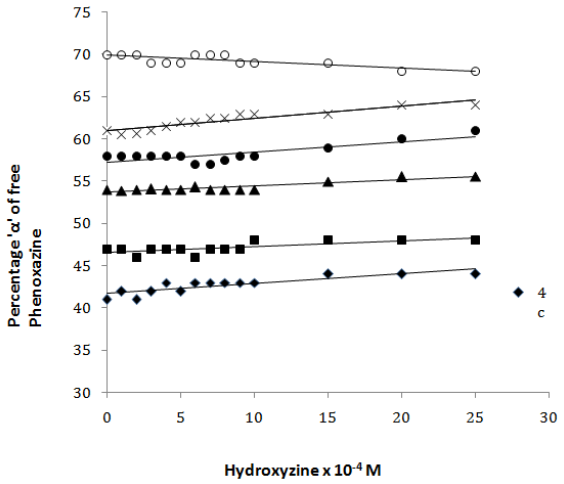


Fig. 5: Influence of hydroxyzine on the binding of phenoxazine derivatives to bovine serum albumin. Ordinate: percentage of free phenoxazine derivative in the albumin solution Abscissa: Total concentration of hydroxyzine (10^{-4}M). All measurements were made in a 1% albumin solution containing 10^{-4}M phenoxazine derivative and varying concentration on hydroxyzine. For statistical evaluation see table 5**. Each point represents the mean value of the two single experiments**

Acetyl salicylic acid competes with the benzene rings of the phenothiazine ring system for binding to BSA[23]. Since acetyl salicylic acid competes for phenothiazine ring system for binding to BSA, the authors have examined the effect of acetyl salicylic acid as a displacing agent to determine whether the benzene rings of phenoxazine modulator are involved in binding to BSA. The displacing experimental data are shown in Fig.6. The experimental data revealed that acetyl salicylic acid displaces phenoxazine derivatives (1C-6C) by 10-25% from their BSA binding sites, strongly suggesting that the benzene rings of phenoxazine derivatives are involved in binding with BSA.

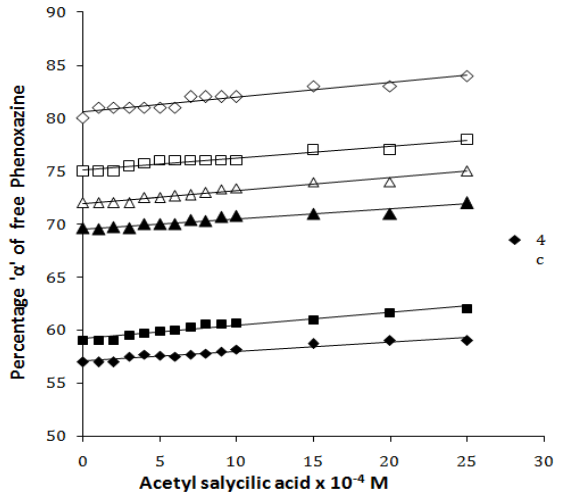


Fig. 6: Displacement of phenoxazine derivatives by acetylsalicylic acid from binding to bovine serum albumin. Ordinate: Percentage of free phenoxazine derivative. Abscissa: total concentration of acetylsalicylic acid (10^{-4}M). All measurements were made in a 1% albumin solution containing one of the phenoxazine derivatives (10^{-4}M) and varying concentrations of acetylsalicylic acid. For statistical evaluation see table 5**. Each point represents the mean value of the two single experiments**

This study is expected to provide important insight into the essence, potential toxicity between drugs and protein in real terms, and can also provide a useful clinical reference for future combination therapy.

Table 6: Minimum inhibitory concentrations of (MIC) of standard antibiotics and MDR modulators (1C-6C)

Phenoxazine/antibiotic	*E. coli* K12MG 1655 ug/ml	*E. coli* ST 58 ug/ml
1C	>181.82	>181.82
2C	>181.82	>181.82
3C	181.82	181.82
4C	181.82	181.82
5C	181.82	181.82
6C	>181.82	181.82
Streptomycin	22.72	90.90
Spectinomycin	22.72	181.82
Gentamycin	11.36	45.45
Kanamycin	22.72	45.45
Benzyl penicillin	90.90	45.45

MIC values are mean of two experiments, each with three replicates.

Effect of phenoxazine modulators on the reversal of bacterial drug resistance

Phenoxazines proved to be a unique class of compounds with prominent biological activities**.** In general, phenoxazines are electron donors and bind by charge-transfer complex formation to target molecules when an electron is supposed to go from the highest filled molecular orbital to the lowest empty orbital of the acceptor molecule on the target. When the phenoxazine acts as an electron donor at the surface of the plasma membrane of the cell or within the lipid bilayer of the plasma membrane, then the electron transfer on the outside will result in depolarization of the membrane. When the phenoxazine acts as an electron donor on the cytoplasmic side of the plasma membrane, hyper-polarization results and membrane-linked processes are inhibited. If the biological activity is actually due to charge-transfer complex formation, then we expect the pharmacological activity of phenoxazines results from their electron donor property.

The main mechanisms whereby the bacteria develop resistance to antimicrobial agents include enzymatic inactivation [39, 40], modification of the drug target (s), and reduction of intracellular drug concentration by changes in membrane permeability or by the over expression of the efflux pumps [40, 41]. With respect to efflux pumps, they provide a self-defense mechanism by which antibiotics are actively removed from the cell. For anti-bacterials, this results in sub-lethal drug concentrations at the active site that in turn may predispose the organism to the development of high-level target-based resistance [40, 42]. Therefore, efflux pumps are viable antibacterial targets and identification and development of potent efflux pump inhibitors is a promising and valid strategy [40, 43] which can restore the susceptibility of resistant strains to antibacterial agents that are substrates of efflux pumps[40, 44].

Among the activities reported for phenoxazines are those that affect the activity of efflux pump P-glycoprotein encoded by the ABCB1 gene of cancer cells that express a MDR phenotype [7-9, 12]. In addition, Thimmaiah *et al.* [45] have shown that the effect of sub-inhibitory concentrations of 2-trifluoromethyl-N^{10}-substituted phenoxazines on plasmid-coded antibiotic resistance in *E. coli.* Phenoxazine treatment resulted in the loss of resistance 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 [45]. 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. The potency of phenoxazines to sensitize the resistant organisms follows the order: butyl>propyl>acetyl derivatives [45].

Further, it is supported that the enhancement of the antibacterial activity of antibiotics against the resistance of *E. coli* K12 MG 1655 and *E. coli* ST 58 by phenoxazines, is associated with the block of the efflux pump, Pgp or P-gp type of efflux pump. Of note that 2,10-disubstitutedphenoxazine derivatives at their non-toxic concentrations were found to be very effective in reversing the resistance of the cancer cells [8,10-12], and therefore, the studies have been extended by the authors to explore the efficacy of six 2-trifluoromethyl-N^{10}-substituted phenoxazines (IC-6C) on the potentiation of antibacterial activity of antibiotics against resistant organisms such as *E. coli* K12 MG 1655 and *E. coli* ST 58 using combinatorial technique (antibiotic+modulator). MIC was determined with serial concentrations of antibiotics in the presence of minimum effective concentration (less than MIC) of phenoxazines. The results are expressed in terms of fold-potentiation (i.e., ratio between the MIC of antibiotic alone and MIC of an antibiotic in the presence of phenoxazine chemosensitizer) which is an index of the efficacy of the modulator for reversing the resistance. The effect of six modulators (1C-6C) on the MIC of five antibiotics (streptomycin, spectinomycin, gentamycin, kanamycin and benzyl penicillin) against two moderately resistant strains of *E. coli* K12 MG 1655 and *E. coli* ST 58 were determined and the results are given in Tables 7-11. The MIC values of six modulators and five antibiotics are given in table 6. On the basis of MIC values of five antibiotics in the absence and presence of each modulator, the fold-potentiation was calculated and the values lie in the range 1 to 32-fold. Compound 4C exhibited maximum modulating effect in the case of kanamycin by 32-fold in the case of *E. coli* K12 MG 1655. The compound 3C increases the activity of streptomycin by 16-fold in *E. coli* ST 58. The compound 4C potentiated the activity of gentamycin by 16-fold in *E. coli* ST 58.

Table 7: Effect of 2-trifluromethyl-N^{10}-substituted phenoxazine modulators on the antibacterial activity of streptomycin against resistant strains

Modulators	*E. coli* K12 MG 1655			*E. coli* ST58		
	Required modulator concentration (µg/ml)	MIC of streptomycin with modulators (µg/ml)	Fold potentiation	Required modulator concentration (µg/ml)	MIC of streptomycin with modulators (µg/m)	Fold-potentiation
1 C	22.72	2.84	8	22.72	45.45	2
2 C	22.72	2.84	8	45.45	11.36	8
3 C	11.36	2.84	8	22.72	5.68	16
4 C	22.72	5.68	4	22.72	11.36	8
5 C	22.72	5.684	4	5.45	90.90	1
6 C	22.72	5.68	4	22.72	11.36	8

MIC values are mean of two experiments, each with three replicates

Table 8: Effect of 2-trifluromethyl-N^{10}-substituted phenoxazine modulators on the antibacterial activity of *spectinomycin* against resistant strains

Modulators	*E. coli* K12 MG 1655			*E. coli* ST58		
	Required modulator concentration (µg/ml)	MIC of spectinomycin with modulators (µg/ml)	Fold-potentiation	Required modulator concentration (µg/ml)	MIC of spectinomycin with modulators (µg/ml)	Fold-potentiation
1 C	22.72	5.68	4	22.72	181.82	1
2 C	45.45	11.36	2	45.45	22.72	8
3 C	45.45	1.42	16	22.72	45.45	4
4 C	22.72	5.68	4	22.72	22.72	8
5 C	22.72	11.36	2	45.45	181.82	1
6 C	22.72	11.36	2	22.72	22.72	8

MIC values are mean of two experiments, each with three replicates

Table 9: Effect of 2-trifluromethyl-N^{10}-substituted phenoxazine modulators on the antibacterial activity of *gentamycin* against resistant strains

Modulators	*E. coli* K12 MG 1655			*E. coli* ST58		
	Required modulator concentration (µg/ml)	MIC of gentamycin with modulators (µg/ml)	Fold-potentiation	Required modulator concentration (µg/ml)	MIC of gentamycin with modulators (µg/ml)	Fold-potentiation
1 C	22.72	2.84	4	22.72	45.45	1
2 C	22.72	2.84	4	22.72	22.72	2
3 C	45.45	11.36	1	22.72	22.72	2
4 C	22.72	2.84	4	45.45	2.84	16
5 C	45.45	2.84	4	45.45	45.45	1
6 C	22.72	2.84	4	45.45	5.68	8

MIC values are mean of two experiments, each with three replicates

Table 10: Effect of 2-trifluromethyl-N^{10}-substituted phenoxazine modulators on the antibacterial activity of *kanamycin* against resistant strains

Modulators	*E. coli* K12 MG 1655			*E. coli* ST58		
	Required modulator concentration (µg/ml)	MIC of kanamycin with modulators (µg/ml)	Fold-potentiation	Required modulator concentration (µg/ml)	MIC of kanamycin with modulators (µg/ml)	Fold-potentiation
1 C	22.72	5.68	4	22.72	22.72	2
2 C	45.45	2.84	8	22.72	45.45	1
3 C	45.45	5.68	4	22.72	45.45	1
4 C	22.72	0.71	32	45.45	2.84	16
5 C	22.72	11.36	2	45.45	45.45	1
6 C	22.72	5.68	4	22.72	5.68	8

MIC values are mean of two experiments, each with three replicates

Table 11: Effect of 2-trifluromethyl-N^{10}-substituted phenoxazine modulators on the antibacterial activity of benzylpenicillin against resistant strains

Modulators	*E. coli* K12 MG 1655			*E. coli* ST58		
	Required modulator concentration (µg/ml)	MIC of benzylpenicillin with modulators (µg/ml)	Fold-potentiation	Required modulator concentration (µg/ml)	MIC of benzyl penicillin with modulators (µg/ml)	Fold–potentiation
1 C	45.45	90.90	1	22.72	45.45	1
2 C	45.45	90.90	1	22.72	45.45	1
3 C	45.45	90.90	1	22.72	45.45	1
4 C	45.45	90.90	1	22.72	45.45	1
5 C	45.45	90.90	1	45.45	45.45	1
6 C	45.45	90.90	1	45.45	45.45	1

MIC values are mean of two experiments, each with three replicates

Further, the authors have attempted to correlate the BSA binding and resistance reversing ability of six modulators (1C-6C) to their structure, physico-chemical properties, pK_a's and $\log_{10}$ P values. Analysis of the data reveal that the modulator 4C is bound to BSA to a greater extent [(β=77.8, m=0.27, k=3.5, K=3.6,ΔF °=25731 (table 3)] and possesses more resistance reversing ability in *E. coli* K12 MG 1655 (up to 32-fold), suggesting that there is a direct correlation between protein binding and reversal of drug resistance.

CONCLUSION

In summary, the results of the investigation suggest that possibly phenoxazines are bound to albumin by hydrophobic interactions of their aromatic rings. The alkyl side chain, particularly butyl chain of phenoxazines intensifies the interaction of phenoxazines with BSA. The displacement of bound phenoxazine derivative by acetyl salicylic acid suggests that benzene rings of phenoxazine derivatives are involved in binding with BSA. Aliphatic side chain between N^{10}of 2-trifluromethyl phenoxazine nucleus and N-atom of the side chain tertiary amine is not in a position to contribute to the binding of the phenoxazine modulators used as indicated by the displacement of bound phenoxazine by hydroxyzine. Again that the longer carbon side chain length (butyl) and pyrrolidine moiety may lie sufficiently close to the surface of the BSA molecule to intensify binding of the phenoxazine molecule by hydrophobic interaction. The compound that binds to a greater extent with protein possesses more activity for reversing of drug resistance. However, there are several studies that demonstrate that efflux pumps such as P-gp have certain optimal substrate characteristics that include hydrophobicity. It is possible that the characteristics for binding to BSA are similar to those that lead to P-gp binding [46].

Since P-glycoprotein (P-gp) is strongly involved in the area of MDR in cancer chemotherapy as well as malarial resistance [47-49], it is speculated on similar line that P-gp or P-gp type of efflux pump could be the most probable candidate for bacterial resistance. It is now clear that P-gp like mechanisms are not restricted to mammalian cancer but are common throughout biota [50, 51]. Bacterial studies are hoped to be of particular importance since these involved primarily in the crucial adverse environment over millions of years and likely to offer interesting insights in physiology as the basis of resistance. Furthermore, in order to unravel the mechanism of action, studies are underway to figure-out the interaction of phenoxazine resistance modifiers with P-gp or P-gp-type of proteins isolated from the resistant bacterial cells. Analogous to phenothiazines, which possess plasmid curing activities rendering the bacterial carrier of the plasmid sensitive to antibiotics, experiments are underway to explore whether phenoxazine treatment would result in the loss of resistance in the plasmid-coded antibiotic resistance in E. coli.

ACKNOWLEDGEMENT

The authors are grateful to the University Grants Commission, Government of India, and the University of Mysore, for financial support (BTS & MNK). This work was also supported by the Department of Science and Technology, Department of Biotechnology, Indian Council of Medical Research, Department of Atomic Energy, and Council of Scientific and Industrial Research, Government of India Grants to KNT.

CONFLICT OF INTERESTS

Declared None

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