Asian Journal of Chemistry Vol. 11, No. 3 (1999), 878–905

Structural Requirements for Activity of Phenoxazines for Reversal of Drug Resistance in Cancer Cells

G.B. EREGOWDA, G. KRISHNEGOWDA, H.N. KALPANA, B.C. CHANNU, C. DASS†, J.K. HORTON‡, P.J. HOUGHTON** and K.N. THIMMAIAH*
Department of Studies in Chemistry
University of Mysore, Manasagangotri, Mysore-570 006, India

In the course of a chemical program aimed at identifying chemically useful modulators of MDR in cancer therapy, a series of twentyone 2-trifluoromethyl-N^{10}-substituted phenoxazines (**1–21**) has been synthesised. The novel 2-trifluoromethylphenoxazine (**1**) was prepared by the pyrolytic condensation of 2-bromophenol and 4-chloro-3-nitrobenzotrifluoride as outlined in Scheme-I. This compound undergoes N-alkylation in the presence of phase transfer catalyst (PTC). Stirring of 2-trifluoromethylphenoxazine with 1-bromo-3-chloropropane or 1-bromo-4-chlorobutane in a two phase system consisting of an organic solvent (benzene) and 6N potassium hydroxide in the presence of tetrabutylammonium bromide leads to the formation of compounds **2** and **9** in good yield. N-(ω-chloroalkyl)- and N-(chloroacetyl) analogues were found to undergo iodide catalysed nucleophilic substitution reaction with various secondary amines including N,N-diethylamine, N,N-diethanol-amine, morpholine, piperidine, pyrrolidine and (β-hydroxyethyl)-piperazine. Purified products were characterized by UV, IR, ^1H and ^{13}C-NMR and mass-spectral data. The lipophilicity expressed in $\log_{10} P$ and pKa of compounds were determined. The effect of **1–21** at 100 μM on the steady-state accumulation of vinblastine (VLB) was studied in $KBCh^R$-8-5 cells and the data revealed that the compounds (**3–8, 10, 12–15**) exhibited a significant VLB uptake enhancing effect (8.3–58.5-fold relative to control) compared to a standard modulator, verapamil (VRP) (7.5-fold). These eleven compounds caused a 1.10-7.82-fold greater uptake of VLB than did a similar concentration of VRP. Comparison of the derivatives for their ability to potentiate the uptake of VLB revealed that they largely follow the order: **N^{10}-propyl > N^{10}-butyl > N^{10}-acetyl** compounds. To determine whether the increase in VLB uptake upon coincubation with **1–21** modulators was due to a slowing of P-gp mediated efflux, $KBCh^R$-8-5 cells were loaded with [^3H] VLB in the absence of modulator and efflux examined in the absence or presence of 100 μM of **8** or VRP. Less than 10% in the absence or about 40% of cell associated VLB in the presence of 100 μM **8** remained at the end of a 2 h efflux period, suggesting that modulator **8**, like VRP, is able to inhibit *p*-glycoprotein (P-gp) mediated efflux. Cytotoxicity was determined and the IC_{10} and IC_{50} values lie respectively in the range 0.1–30.9 μM and 2.1–70.9 μM for $KBCh^R$-8-5 cells. Substitution of hydrogen by CF_3 in

†Department of Chemistry, University of Memphis, Tennesse 38152, USA.
‡Laboratory of Structural Biology, National Institute of Environmental Health Science, MD F3-01, P.O. Box 12233, 111, T.W. Alexander Drive, Research Triangle Park, NC 27709, USA.
**Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101, USA.

C-2 of phenoxazine ring caused a greater enhancement in the antiproliferative protency by 1.1–3.3-fold for $KBCH^R$-8-5 cells than their counterparts, presumably due to increased hydrophobicity. Compounds at IC_{10} were evaluated for their efficacy to modulate the cytotoxicity of VLB in $KBCh^R$-8-5 cells and compounds **3, 5, 11** and **13**, like VRP, were able to completely reverse the 24-fold resistance of $KBCh^R$-8-5 cells to VLB. The structural features for reversal of MDR seem to include a hydrophobic phenoxazine ring with a $—CF_3$ in C-2 position and a tertiary amino group at a distance of three or four carbon chain from the tricyclic ring. Examination of the relationship between partition coefficient and cytotoxicity or anti-MDR activity showed no correlation suggesting that lipophilicity is not the sole determinant of potency for biological activity.

INTRODUCTION

Resistance of cytotoxic drugs represents a major problem in the treatment of cancer. One cause of the lack of sensitivity of tumor cells to cytotoxic drugs is the overexpression of a membrane glycoprotein called *p*-glycoprotein (P-gp)[1–3]. This transmembrane protein functions as an ATP-dependent efflux pump[4] for a large variety of structurally and functionally diverse drugs, such as anthracyclines, epipodophyllotoxines, actinomycin D, vinca alkaloids, colchicine and taxol. Presently available data suggest that P-gp expression contributes to chemical resistance in human tumors[5–8]. Substances capable of reestablishing toxicity of chemotherapeutic agents *via* inhibition of this efflux pump have been described in the literature. These include calcium channel blockers[9], calmodulin inhibitors[10], antiarrythmics[11], antimalarials[12], lysosomotropic agents[13], steroids[14], anti-estrogens[15] and cyclic peptide antibiotics[16]. All of these compounds are effective modulators *in vitro*. They lower the IC_{50} values of a variety of drugs included in the MDR family and they increase intracellular drug concentrations in resistant cells. The mechanism responsible for this reversal of resistance is believed to be competition between the modulator and cytotoxic drug for binding to the ATP-dependent efflux pump, P-gp[17, 18]. The clinical utility of any modulator, however, depends not only on its ability to reverse drug resistance at low concentrations but also on whether it has a low toxicity *in vivo*. The cardiac toxicity seen during the clinical evaluation of VRP as a chemosensitizing agent pointed out the need for less toxic modulators[19, 20]. Two other antiarrythmic drugs, quinidine and amiodarone have also entered clinical trials as chemosensitizing agents[21] and both drugs have produced a number of adverse clinical side effects[22]. While a number of pharmacological agents have been shown to reverse MDR *in vitro*, there remains a need to identify more potent, more specific and less toxic chemosensitizers for clinical use.

Thimmaiah *et al.*[23] have reported that phenoxazine potentiated the uptake of vinca alkaloids in MDR GC_3/cl and $KBCh^R$-8-5 cells to a greater extent than VRP. However, it was less effective in sensitizing MDR cells, in part, due to its instability in culture medium. Subsequently, twentyone phenoxazine derivatives were synthesised and examined for their ability to enhance the uptake of VLB and VCR in GC_3/cl and $KBCh^R$-8-5 cells. The results revealed that substitution on the phenoxazine ring at position N^{10} was associated with an increase in

antiproliferative and anti-MDR activities. The present study has been undertaken to further improve the chemosensitising potency of 2,10-disubstituted phenoxazine derivatives. Therefore, we have synthesised twentyone phenoxazine derivatives which were structurally modified at the C-2 position and tested for their anti-MDR activity *in vitro* systems for understanding the novel mechanisms of MDR.

EXPERIMENTAL

Reactions were monitored by TLC. Column chromatography utilized silica gel Merck grade (230–400 mesh, 60 Å). Melting points were recorded on a Kofler hot-stage with microscope and are uncorrected. UV spectra were recorded in MeOH on a JASCO Model 610 spectrophotometer. IR spectra were recorded on a Perkin-Elmer Model 1320 spectrophotometer as KBr pellets. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee, USA. Found values are within ±0.4% of theoretical values unless otherwise noted. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solution in a 5-mm tube on a JEOL CPF-270 Fourier transform spectrometer with tetramethylsilane as internal standard. Chemical shifts are expressed as "δ" (ppm) values. The spectrometer was internally locked to the deuterium frequency of the solvent.

Liquid secondary ionization mass spectrometry (LSIMS) was used to obtain the molecular weight information. The positive-ion LSIMS spectra of the compounds were obtained using Autospec Q (VG Analytical, Manchester, UK) hybrid tandem mass spectrometer of E_1BE_2-qQ geometry (where E is an electric sector, B a magnetic sector, q an r.f.-only quadrupole, and Q a quadrupole mass analyzer). Only the front end (*i.e.*, E_1BE_2) was used to obtain the conventional magnet scan mass spectra. The data acquisition and manipulation were under the control of Digital Vax Station 3100-based Opus Software. A few μg of those compounds was dissolved in *m*-nitrobenzyl alcohol (used as liquid matrix) and 0.5–1.0 μL was applied to the LSIMS probe tip. The matrix/sample mixture was bombarded with a beam of 30–35 keV Cs+ ions. The secondary ions excited the ion source at a potential of 8 kV and were analyzed by scanning the magnetic field.

All the chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. VRP hydrochloride and colchicine were purchased from Aldrich Chemical Co. VLB sulfate was from Cetus Corporation (Emeryville, CA). [G-^{3}H] VLB (sp. act. 9 Ci/mmol) was from Moravek (Brea, CA). Antibiotic free RPMI-1640 and DMEM medium powder with glutamine and without sodium bicarbonate from Gibco BRL (Grand Island, NY) and Bio Whittaker (Walkersville, MD) were purchased.

Synthesis

2-Bromo-2′-nitro-4′-trifluoromethyldiphenylether **(I)**: 34.7 mL (53.43 g, 0.2368 mol) of 4-chloro-3-nitrobenzotrifluoride and 27 mL (40.28 g, 0.233 mol) of 2-bromophenol and 3 mL of water were stirred at room temperature for about 15 min. Then 16.64 g of potassium hydroxide pellets were added slowly in three

portions with constant stirring. The mixture was refluxed at 105°C for 4 h. The reaction mixture was cooled to room temperature and added dilute sodium hydroxide solution (sufficient to make it basic). Then, the reaction mixture was extracted with benzene three times. The benzene layer, after washing three times with dilute sodium hydroxide, was dried over anhydrous sodium sulfate and rotavaporated to give an oily product, which on treatment with petroleum ether separated out as a crude yellowish brown solid (**I**) (60.35 g, 71%). 1 g of crude product was subjected to column chromatography on silica gel with chloroform-petroleum ether (3.5 mL + 6.5 mL) eluted pure (**I**).

2′-Amino-2-bromo-4′-trifluoromethyldiphenylether (**II**): 65.0 g (0.179 mol) of compound (**I**) was taken in a round-bottom flask to which added 300 mL of distilled water. Initially it was heated to 50–60°C when the solid melted into an oil. Then added slowly 78.45 g of iron filings into the flask and started refluxing. While refluxing, 149.52 mL (157 g) of glacial acetic acid was added from the droping funnel drop by drop over a period of 8 h. Then cooled the contents and extracted with benzene several times. The organic layer was washed with water three times, dried over anhydrous sodium sulfate and rotavaporated to give the crude product (**II**) (45 g, 76%) as a brown oil. 1 g of crude product (**II**) was chromatographed on silica gel with chloroform + petroleum ether (3.0 mL + 7.0 mL) to get the pure compound (**II**) in the form of yellow oil.

2-Trifluoromethyl phenoxazine (**1**): 45.0 g of (**II**) was stirred at 140–160°C in 9.5 mL of formic acid (11.07 g) for 1 h, when 2-bromo-2′-formamido-4′-trifluoromethyl diphenylether (**III**) was formed as evidenced by TLC. The residual water and formic acid present were removed by distillation and remaining volatiles were taken off the compound (**III**) by applying high vacuum overnight, when no pungent smell due to formic acid was perceived. The formamido derivative (**III**) was mixed with 12.55 g of anhydrous K_2CO_3, 0.74 g of $CuCO_3$ and 60 mL of anhydrous *p*-xylene and refluxed at 155–160°C over a water separator for 16 h to give N-formyl-2-trifluoromethyl phenoxazine (**IV**) as monitored by TLC. A solution of 5.90 g of sodium hydroxide in 37 mL of water was added and the contents were refluxed for 2 h. Enough water and benzene were added to dissolve the solid and extracted completely. The benzene layer was washed with water three times, dried over anhydrous sodium sulfate and rotavaporated to give crude solid. This dark solid was washed several times with *n*-hexane which removes all the other impurities. Sample of 2-trifluoromethyl-phenoxazine (**1**) (24 g, 70%) was obtained which was further purified by column chromatography, m.p. 148–150°C; UV-λ_{max} (ε) (MeOH) 241 (41472), 319 (8832) nm; IR 3400, 2924, 2855, 1633, 1591, 1508, 1456, 1344, 1307, 1234, 1203, 1163, 1126, 1107, 1070, 935, 925, 868, 829, 725 cm^{-1}; MS (m/z) 251 [$M^{+\bullet}$].

10-(3′-Chloropropyl)-2-trifluoromethylphenoxaxine (**2**): Method (A): 1.0876 g (0.035 mol) of sodium amide was taken in 100 mL of liquid ammonia to which added 7.0 g (0.028 mol) of (**1**). After stirring for 45 min, 4.723 g (0.03 mol, 3.0 mL) of 1-bromo-3-chloropropane was added slowly with constant stirring. After 2 h, ammonia was allowed to evaporate and solid ice pieces were added carefully followed by cold water. When the reaction ceased, the mixture

was extracted thrice with ether. The ether fraction was washed three times with water, dried over anhydrous sodium sulfate and evaporated.

Method (B): 7.0 g (0.028 mol) of **1** was dissolved in 40 mL of benzene and added 180 mL of 6 N KOH and 4.51 g of tetrabutylammonium bromide (0.014 mol) to it. The reaction mixture was stirred at room temperature for 30 min. Added 1-bromo-3-chloropropane (0.038 mol) [or 1-bromo-4-chlorobutane (0.04 mol) for compound **9**] slowly into the reaction mixture and stirred at room temperature for 24 h. Benzene was evaporated and the aqueous layer extracted with ether. The ether layer was washed with water and organic layer dried over anhydrous sodium sulfate and rotavaporated.

The residue was chromatographed on silica gel. Petroleum ether-chloroform (6.5 mL + 3.5 mL) eluted pure **2** (6.2 g, 68%) as pure crystalline product. m.p. 68°C; UV-λ_{max} (ε) (MeOH) 243 (13043), 325 (2559) nm; IR 2928, 1632, 1595, 1498, 1444, 1371, 1283, 1279, 1259, 1136, 1115, 1072, 927, 852, 816, 744, 646 cm^{-1}; MS (m/z) 327 [$M^{+\bullet}$].

10-[3′-(*N-Diethylamino)propyl*]-2-*trifluoromethyl phenoxazine* (**3**): 1.0 g (3.053 mmol) of **2** was dissolved in 100 mL of anhydrous acetonitrile, 1.5 g of KI and 2.5 g of K_2CO_3 were added and refluxed for 30 min. Then added 1.1 g (15.09 mmol, 1.0 mL) of N,N-diethylamine and refluxed for 48 h until a substantial amount of the product was formed as evidenced by TLC. The contents were cooled, diluted with water and extracted with ether. The ether layer was washed with water thrice, dried over anhydrous sodium sulphate and evaporated to give an oily product. The oily residue was chromatographed on silica gel. Ethylacetate-methanol (9 mL + 1 mL) eluted pure (**3**) as pure light yellow oil. An ether solution of the free base was treated with ethereal hydrochloride to give hydrochloride salt which was dried under high vacuum to get pure solid (**3**) (1.04 g, 85%). m.p. 164°C; UV-λ_{max} (ε) (MeOH) 242 (9529), 323 (4833) nm; IR 2900, 2600, 1630, 1593, 1493, 1442, 1371, 1319, 1265, 1161, 1118, 1078, 939, 846, 816, 752 cm^{-1}; MS (m/z) 365 [M + H]$^+$.

10-[3′-[*N-Bis(hydroxyethyl)amino]propyl*]-2-*trifluoromethyl phenoxazine* (**4**): 1.06 g (3.26 mmol) of **2**, 1.5 g of KI, 2.5 g of K_2CO_3 and 1.2 mL (1.28 g, 9.83 mmol) of N,N-diethanolamine were refluxed for 24 h and followed the rest of the procedure used for **3**. Pure base obtained was kept in the freezer for overnight to get the light yellow solid **4** (1.0 g, 82%). m.p. 44°C; UV-λ_{max} (ε) (MeOH) 244 (37242), 325 (8051) nm; IR 3290, 2900, 1581, 1510, 1412, 1377, 1340, 1275, 1180, 1080, 929, 877, 719 cm^{-1}; MS (*m/z*) 396 [$M^{+\bullet}$].

10-(3′-*N-Morpholinopropyl*)-2-*trifluoromethyl phenoxazine* (**5**): The procedure used for **4** was repeated with 1.0076 g (3.07 mmol) of **2**, 1.52 g of KI, 2.022 g of K_2CO_3 and 1.064 g of (12.2 mmol, 1.07 mL) morpholine. The oily product was purified by column chromatography using ethylacetate-petroleum ether (5 mL + 5 mL) as solvent system. The light yellow oil was kept in the freezer for about 2 h to get a yellow solid of (**5**) (1 g, 86%) m.p. 66°C; UV-λ_{max} (ε) (MeOH) 244 (31640), 326 (6389) nm; IR 3067, 1608, 1551, 1462, 1358, 1273, 1205, 1088, 1037, 989, 925, 845, 729, 642 cm^{-1}; MS (m/z) 378 [$M^{+\bullet}$].

10-(3′-*N-Piperidinopropyl*)-2-*trifluoromethyl phenoxazine* (**6**): Repeated the procedure used for **4** with 1.09 g (3.32 mmol) of **2**, 1.5 g of KI, 2.5 g of

K_2CO_3 and 1.2 g (14.0 mmol, 1.39 mL) of piperidine. Purification by column chromatography gave the free base in the form of an oil which was converted into hydrochloride salt **6** (1.12 g, 82%). m.p. 208–210°C; UV-λ_{max} (ε) (MeOH) 238 (8625), 323 (7456) nm; IR 2928, 2635, 1630, 1593, 1441, 1369, 1315, 1268, 1230, 1132, 1116, 1078, 1045, 939, 879, 845, 754 cm^{-1}; MS (m/z) 377 $[M + H]^+$.

10-(3′-*N-pyrrolidinopropyl*)-2-*trifluoromethyl phenoxazine* (**7**): The experimental procedure used for **4** is applicable with 1.0 g (3.05 mmol) of **2**, 1.5 g of KI, 2.5 g of K_2CO_3 and 1.0 g (14.08 mmol, 1.2 mL) of pyrrolidine. The oily compound was chromatographed on the silica gel to get the pure product **7**. By adding ethereal hydrochloride to the ether solution of the free base, hydrochloride salt of **7** (1.2 g, 98%) was obtained. m.p. 218°C; UV-λ_{max} (ε) (MeOH) 238 (13175), 324 (6822) nm; IR 2855, 1600, 1493, 1441, 1369, 1317, 1263, 1157, 1078, 935, 885, 846, 754, 673 cm^{-1}; MS (m/z) 363 $[M + H]^+$.

10-[3′-[(β-*hydroxyethyl*)*piperazino*]*propyl*]-2-*trifluoromethyl phenoxazine* (**8**): Compound **8** as its salt (1.5 g, 76%) was obtained by following the procedure of **4** with 1.3 g (3.96 mmol) of **2**, 1.5 g of KI, 2.5 g of K_2CO_3 and 2.15 g (16.51 mmol, 2.03 mL) of (β-hydroxyethyl)piperazine. The free base was purified by column chromatography to give a light green syrupy oil. m.p. not sharp; UV-λ_{max} (ε) (MeOH) 238 (15225), 325 (7107) nm; IR 3262, 2644, 2436, 1634, 1595, 1498, 1448, 1375, 1325, 1277, 1267, 1149, 1134, 1096, 1080, 1041, 968, 850, 808, 736, 646 cm^{-1}; MS (m/z) 422 $[M + H]^+$.

10-(4′-*Chlorobutyl*)-2-*trifluoromethyl phenoxazine* (**9**): Compound **9** (9.5 g, 93%) in the pure form was prepared following the procedure used for **2** with 7.5 g (0.03 mol) of **1**, 1.6 g (0.041 mol) of sodium amide and 5.487 g (0.032 mol, 3.7 mL) of 1-bromo-4-chlorobutane. m.p. 42°C; UV-λ_{max} (ε) (MeOH) 245 (21511), 324 (9417) nm; IR 2922, 1632, 1593, 1493, 1444, 1371, 1321, 1277, 1223, 1134, 1115, 1075, 935, 848, 748, 646 cm^{-1}; MS (m/z) 341 $[M^{+\bullet}]$.

10-[4′-[*N-Diethylamino*] *butyl*]-2-*trifluoromethyl phenoxazine* (**10**): The procedure of **3** was followed wth 1.0 g (2.92 mmol) of **9**, 1.5 g of KI, 2.5 g of K_2CO_3 and 1.26 g (17.22 mmol, 1.8 mL) of N,N-diethylamine to get **10**. The light yellow oil was purified by column chromatography by washing the column with ethylacetate and methanol mixture and converted into hydrochloride salt of **10** (1.13 g, 93%). m.p. 162–165°C; UV-λ_{max} (ε) (MeOH) 239 (7648), 324 (6788) nm; IR 2936, 2561, 1632, 1595, 1496, 1444, 1321, 1261, 1205, 1161, 1115, 1076, 1045, 956, 916, 856, 810, 740, 650 cm^{-1}; MS (m/z) 379 $[M + H]^+$.

10-[4′-[*N-Bis*(*hydroxyethyl*)*amino*]*butyl*]2-*trifluoromethyl phenoxazine* (**11**): 1.26 g (3.68 mmol) of **9**, 1.5 g of KI, 2.0 g of K_2CO_3 and 1.28 g (1.20 mL, 9.83 mmol) of N,N-diethanolamine were refluxed and processed according to the procedure used for **3**. Purified compound **11** as hydrochloride salt (1.21 g, 80%) was obtained. m.p. 94–96°C; UV-λ_{max} (ε) (MeOH) 243 (37881), 325 (7999) nm; IR 3350, 2900, 1630, 1593, 1495, 1448, 1373, 1325, 1275, 1263, 1041, 1022, 962, 931, 854, 819, 750, 679 cm^{-1}; MS (m/z) 412 $[M^{+\bullet}]$.

10-(4′-*N-Morpholinobutyl*)-2-*trifluoromethyl phenoxazine* (**12**): 1.037 g (3.03 mmol) of **9**, 1.62 g of KI, 2.0 g K_2CO_3 and 1.06 mL (1.058 g, 12.41 mmol) of morpholine in 100 mL of acetonitrile were refluxed for 24 h and worked up

according to the protocol used for **11**. The product was purified by column chromatography and pure base in the form of solid **12** (1.0 g, 84%) was obtained. m.p. 66°C; UV-λ_{max} (ε) (MeOH) 243 (42389), 325 (9475) nm; IR 2860, 2812, 1630, 1593, 1496, 1442, 1367, 1277, 1115, 1076, 1043, 999, 895, 856, 816, 752, 625 cm^{-1}; MS (*m/z*) 392 [$M^{+\bullet}$].

10-(4′-*N-Piperidinobutyl*)-2-*trifluoromethyl phenoxazine* (**13**): The experimental steps used for **4** were repeated by taking 1.1 g (3.22 mmol) of **9**, 1.5 g of KI, 2 g of K_2CO_3 and 1.4 g (16.44 mmol, 1.61 mL) of piperidine. The crude product was chromatographed on silica gel to get pure light yellow compound **13** which was converted into hydrochloride salt (1.3 g, 94%). m.p. not sharp; UV-λ_{max} (ε) (MeOH) 237 (9179), 324 (6650) nm; IR 2934, 2866, 2486, 1632, 1593, 1446, 1373, 1323, 1261, 1230, 1109, 1080, 1043, 1016, 983, 916, 850, 808, 738, 626 cm^{-1}; MS (m/z) 391 $[M + H]^+$.

10-(4′-*N-Pyrrolidinobutyl*)-2-*trifluoromethyl phenoxazine* (**14**): The experimental method employed for **4** was used with 1.2 g (3.5 mmol) of **9**, 1.5 g of KI, 2.5 g of K_2CO_3 and 2.2 g (16.9 mmol, 2.58 mL) of pyrrolidine as reactants. The purified oily compound was converted into hydrochloride salt of **14** (1.3 g, 90%). m.p. 178–180°C; UV-λ_{max} (ε) (MeOH) 245 (10125), 323 (7180) nm; IR 2928, 2667, 2457, 1633, 1595, 1496, 1467, 1444, 1373, 1323, 1299, 1226, 1161, 1118, 1076, 1024, 943, 914, 854, 744, 650 cm^{-1}; MS (m/z) 377 $[M + H]^+$.

10-[4′-[(β-*Hydroxyethyl)piperazino]butyl*]-2-*trifluoromethyl phenoxazine* (**15**): The method employed for **4** was used with 1.3 g (3.8 mmol) of **9**, 1.5 g of KI, 2.5 g of K_2CO_3 and 2.2 g (16.9 mmol, 2.1 mL) of β-hydroxyethyl) piperazine to get **15** (1.5 g, 96%). m.p. 73°C; UV-λ_{max} (ε) (MeOH) 223 (21088), 326 (5668) nm; IR 3300, 2816, 1630, 1593, 1496, 1441, 1365, 1321, 1253, 1230, 1163, 1113, 1078, 1041, 928, 846, 810, 758, 852 cm^{-1}; MS (m/z) 411 [$M^{+\bullet}$].

10-(*Chloroacetyl*)-2-*trifluoromethyl phenoxazine* (**16**): To a solution of 6.0167 g (0.02397 mol) of **1** dissolved in 80 mL of acetonitrile containing 150 mL of ether was added 7.5 mL (10.635 g, 0.094 mol) of chloroacetylchloride slowly with stirring. The reaction mixture was stirred at about 40°C for 15 h and evaporated to give pinkish white solid which was purified by column chromatography to give pure **16** (7.6 g, 97%). m.p. 136°C; UV-λ_{max} (ε) (MeOH) 229 (12458), 288 (4733), 330 nm; IR 2959, 1686, 1624, 1584, 1483, 1404, 1329, 1271, 1217, 1174, 1111, 1068, 1030, 983, 887, 835, 771, 675 cm^{-1}; MS (m/z) 327 [$M^{+\bullet}$].

10-[(*N-Diethylamino)acetyl*]-2-*trifluoromethyl phenoxazine* (**17**): 1.2 g (3.66 mmol) of **16** was dissolved in 100 mL of anhydrous acetonitrile and to this solution added 1.5 g of KI and 1 g (13.7 mmol, 1.4 mL) of N,N-diethylamine. The reaction mixture was refluxed for 1 h when substantial amount of the product was formed. The reaction mixture was processed according to the procedure used for **4** and purified the oily product by column chromatography. Pure **17** was converted into hydrochloride salt (1.2 g, 88%). m.p. not determined due to hygroscopic nature; UV-λ_{max} (ε) (MeOH) 227 (13816), 288 (8942) nm; IR 2900, 1691, 1622, 1589, 1489, 1334, 1275, 1219, 1167, 1120, 1066, 1035, 981, 831, 760, 638 cm^{-1}; MS (m/z) 365 $[M + H]^+$.

10-(*N-Morpholinoacetyl*)-2-*trifluoromethyl phenoxazine* (**18**): The procedure used for **17** was repeated with 1 g (3.06 mmol) of **16**, 1.5 g of KI and 1.45 g

(16.55 mmol, 1.44 mL) of morpholine. The residue was purified by column chromatography and pure compound **18** was obtained as a white solid (1.2 g, 88%). m.p. 123°C; UV-λ_{max} (ε) (MeOH) 231 (14289), 292 (7864), 333 nm; IR 3042, 2826, 1678, 1587, 1485, 1458, 1333, 1315, 1271, 1199, 1167, 1112, 1068, 987, 868, 762, 667 cm^{-1}; MS (m/z) 379 $[M + H]^+$.

10-(N-*Piperidinoacetyl*)-2-*trifluoromethyl phenoxazine* **(19)**: The experimental procedure used for **17** was employed with 1.0274 g (3.14 mmol) of **16**, 1.5 g of KI and 1.33 g (15.5 mmol, 1.55 mL) of piperidine to get **19** (1 g, 89%). m.p. 79°C; UV-λ_{max} (ε) (MeOH) 240 (12236), 278 (3512), 330 nm; IR 3022, 2900, 1626, 1585, 1516, 1394, 1314, 1275, 1160, 1011, 898, 860, 798, 756, 638 cm^{-1}; MS (m/z) 377 $[M^{+\bullet}]$.

10-(*N-Pyrrolidinoacetyl*)-2-*trifluoromethyl phenoxazine* **(20)**: 1.0 g (3 mmol) of **16**, 1.5 g of KI and 1.02 g (14.3 mmol, 1.2 mL) of pyrrolidine were refluxed and processed according to the procedure used for **17**. Purification by column chromatography afforded the pure compound **20** (1 g, 90%), m.p. 100°C; UV-λ_{max} (ε) (MeOH) 238 (10118), 290 (6088) 325 nm; IR 2960, 1691, 1624, 1587, 1487, 1437, 1335, 1275, 1257, 1201, 1124, 1068, 1034, 833, 758, 636 cm^{-1}; MS (m/z) 363 $[M + H]^+$.

10-[[(β-*Hydroxyethyl)piperazino]acetyl*]-2-*trifluoromethyl phenoxazine* **(21)**: To a solution of 1.0 g (3 mmol) of **16** in 100 mL of anhydrous acetonitrile, 1.5 g of KI and 1.6 g (12.3 mmol, 0.937 mL) of β-hydroxyethyl) piperazine were added. The reaction mixture was refluxed for 1 h and processed as above. The solid **21** was purified by column chromatography and dried under high vacuum (1.2 g, 94%). m.p. 168°C; UV-λ_{max} (ε) (MeOH) 241 (11421), 277 (6818), 330 nm; IR 3390, 2900, 1689, 1624, 1587, 1487, 1437, 1334, 1275, 1259, 1167, 1122, 1068, 983, 960, 829, 760, 638 cm^{-1}; MS (m/z) 422 $[M + H]^+$.

Determination of pK_a values

The pK_as were determined by a previously published method[24]. Briefly, **10** mL of an approximately 10^{-3} M solution of each modulator (**1–21**) was titrated with 1 M hydrochloric acid or sodium hydroxide using a combination glass electorde to measure pH.

Measurement of lipophilicity

HPLC grade 1-octanol was presaturated with PBS and aqueous phase PBS was saturated with 1-octanol before use. The compounds to be tested were each dissolved in 1-octanol/buffer phase at a final concentration of 1×10^{-4} M and an equal volume of buffer/1-octanol was added. The tubes were then inverted continuously for 15 min; control experiments confirmed that equilibrium was reached within this time. The final concentration of compound in both aqueous and octanol phases was measured by UV-spectrophotometry. The partition coefficient, P was determined by dividing the concentration of the 2-trifluoromethyl phenoxazine derivative in 1-octanol by the concentration in the aqueous phase.

Cell lines and cell culture

Human epidermoid carcinoma KB-3-1 cells and a colchicine-selected MDR variant, $KBCh^R$-8-5 cells cross-resistant to vincristine (45-fold) and VLB (~ 10-fold) were grown in monolayer culture at 37°C in antibiotic-free Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and L-glutamine in a humidified atmosphere of 10% CO_2 in air. The resistance of $KBCh^R$-8-5 cells was maintained by culturing them with 10 ng/mL of colchicine.

Accumulation studies

Cells (2×10^6 cells) were plated and incubated overnight at 37°C to attach to plastic. Medium was aspirated and cells were washed with (2×2 mL) PT buffer (120 mM NaCl, 20 mM Tris-base, 3 mM K_2HPO_4, 10 mM glucose, 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4). Monolayers were incubated at room temperature for 10 min in PT buffer prior to aspiration and adding 1 mL of serum-free RPMI-1640 HEPES buffer (10.4 g RPMI-1640 medium in one litre of 25 mM HEPES, pH 7.4) containing 55.6 nM [^{3}H] vinblastine with or without 100 μM of VRP or 2-trifluoromethyl-N^{10}-substituted phenoxazines dissolved in water and DMSO (final culture concentration < 0.1% DMSO). After 2 h of incubation at room temperature, medium was rapidly aspirated to terminate the drug accumulation and monolayers were washed four times with ice-cold PBS (g/L: NaCl 8.0; $Na_2HPO_4 \cdot 12H_2O$ 2.9; KCl 0.2; KH_2PO_4 0.2) buffer and drained. To each dish 1 mL of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) was added. After 1 min, monolayers were triturated to give a uniform suspension of cells and radioactivity in 0.75 mL was determined by scintillation counting. Cell number per dish was determined on 200 μL of suspension using the method of Butler[25] and amount of intracellular VLB was determined.

Measurement of VLB efflux

Cells (2×10^6/dish) were plated and incubated overnight at 37°C in CO_2 incubator to attach to plastic. Medium was removed and monolayer cells were washed once with 3 mL of the same buffer and incubated with 1 mL of serum free RPMI-1640 Hepes buffer, pH 7.4, containing 55.6 nM [^{3}H] VLB and 100 μM modulator **8** or VRP for 2 h at room temperature. Drug solutions were aspirated and 3 mL of the same buffer without or with modulator **8** or VRP at 100 μM were added and incubated for 2 h at room temperature. The medium was aspirated from each dish and the cells were washed four times in ice-cold PBS and drained. Cells were harvested and radioactivity per dish was calculated as described above and percentage of VLB remaining in the cells after 2 h was calculated.

Inhibition of cellular proliferation

KB-3-1 or KB-Ch^R-8-5 cells were plated in triplicate at a density of 1000 cells per well in 6-well flat-bottom tissue culture plates. After 24 h at 37°C in an atmosphere of 5% CO_2/95% air, medium was replaced with 3 mL of fresh medium containing phenoxazine compounds (**1–21**) at concentrations ranging from

0–100 μM in DMSO (final culture concentration < 0.1% DMSO) and cells were incubated at 37°C for a further 7 days. The medium was aspirated and cells were washed once with 2 mL of 0.9% sodium chloride and dried overnight. Colonies were stained with 1 mL of 0.1% crystal violet followed by washing twice with distilled water and dried overnight. The colony area was quantitated using an automated ARTEK Model 880 colony counter. The IC_{10} and IC_{50} values were determined from concentration-percent cell survival curves and were defined as the concentrations required for 10% and 50% reduction in colonies compared to controls.

Effect of phenoxazine MDR modulators on *in vitro* cytotoxicity of vinblastine

To determine the effects of the modulators on the cytotoxicity of VLB, KB-3-1 or $KBCh^R$-8-5 cells were treated with serial dilutions of VLB (0–100 nM) in the absence or presence of IC_{10} concentrations of modulators. After incubation for 7 days at 37°C, colonies were enumerated as described above. IC_{50} values were determined as previously described and the fold-potentiation was calculated by dividing the IC_{50} for VLB in the absence of modulator by the IC_{50} in the presence of modulator.

RESULTS AND DISCUSSION

Synthesis

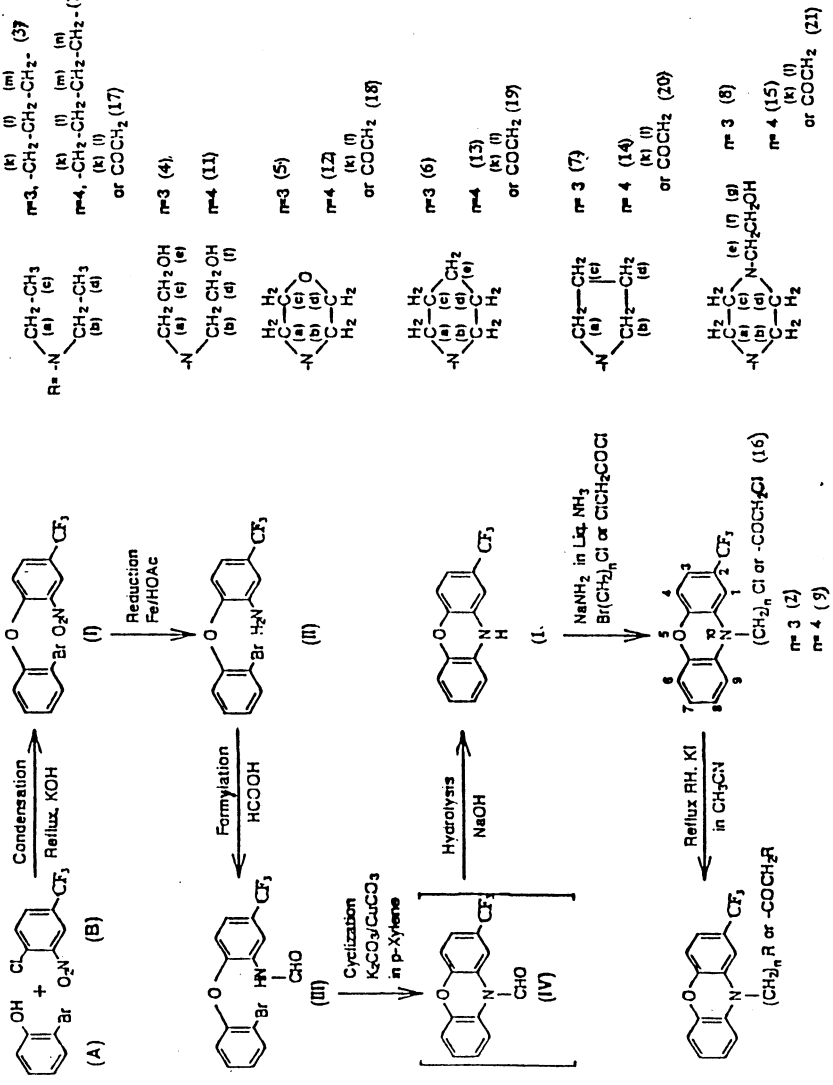
The anti-MDR[26] and other pharmacological properties of phenoxazines[27] prompted us to prepare a series of 2-trifluoromethylphenoxazines for improved anti-MDR activity. The classic method for preparing parent phenoxazine involving the condensation of catechol with *o*-aminophenol necessitated the pressurised reactions and yields were erratic. Weis *et al.*[28] were interested in finding new conditions for the cyclization of substituted 2-(2-chlorophenoxy) anilines, whereby the ring closure should occur with the elimination of hydrogen chloride leading to the formation of variously substituted phenoxazines. But, it was obvious from previous experiments that the normal ring closure between an amino group and a halogen atom, occupying the 2- and 2′-positions, respectively, of phenylether moiety, with the loss of hydrogen halide, did not represent a practicable synthetic procedure. Although, several synthetic routes to phenoxazines have been reported, the yields were very less and, therefore, a new method to acheive the synthetic goal which is simple and efficient, while also unable to large scale preparation has been sought in the present work.

The novel 2-trifluoromethylphenoxazine (**1**) and its derivatives (**2–21**) were prepared according to the synthetic route outlined in Scheme 1. Compound (**1**) was prepared by the pyrolytic condensation of 2-bromophenol (**A**) and 4-chloro-3-nitrobenzotrifluoride (**B**) to form 2-bromo-2′-nitro-4′-trifluoromethyldiphenylether (**I**). The reactants were heated preferably at reflux in the presence of water and potassium hydroxide. This diphenylether thus formed was reduced chemically with iron filings and glacial acetic acid under reflux conditions to form 2′-amino-2-bromo-4′-trifluoromethyldiphenylether (**II**). An attempted cyclization of (**II**)

with sodium amide in boiling *p*-xylene for 19 h failed to give 2-trifluoromethylphenoxazine (**1**); only (**II**) was recovered in 60% yield. (**II**) was also recovered, almost quantitatively, when its cyclization was attempted with potassium carbonate in boiling butanol for 24 h. However, the cyclization of (**II**) was effected when it was heated under reflux in N,N-diemthylformamide with potassium carbonate and a trace of copper powder under dry nitrogen. But the yield of (**1**) was very less (only 39%). However, modification of this cyclization has been accomplished through the N-formyl derivative (**III**), which enhanced the yield of (**1**) to 70%. The amino derivative (**II**) was N-formylated preferably as a melt with a concentrated solution of formic acid (90.8%). The formylation in aqueous solution was advantageously carried out at temperatures from about 140–160°C. The resulting 2-bromo-2′-formamido-4′-trifluoromethyldiphenylether (**III**) intermediate was then cyclized by heating with one molar equivalent or preferably an excess of alkali earth carbonate (potassium carbonate) preferably in the presence of copper catalyst such as cupric carbonate. The reaction was run in a high boiling aromatic solvent, *p*-xylene, which distills azeotropically with water. Intramolecular cyclization of the diphenylether (**III**) proceeds as the cyclization reaction was run at elevated temperatures from 155–165°C for 16 h. Removal of water from the reaction mixture as it was formed enhanced the yield. The N-formyl derivative (**IV**) which was formed by the cyclization reaction was hydrolysed with sodium hydroxide solution to give 2-trifluoromethylphenoxazine (**1**). In general, it was preferred to carry out the reaction without isolating the N-formyl intermediate.

The weakly basic nature of nitrogen atom of the phenoxazine nucleus, in general, resists phenoxazine to undergo N-alkylation with alkyl halides. However, it can be achieved in the presence of basic condensing agents like sodium amide or sodium hydride. The general procedure for preparing N-alkyl derivative consists of condensation of phenoxazines with requisite alkyl halide in the presence of a strong acid binding agent like sodium amide either in liquid ammonia or anhydrous aromatic solvents such as toluene or benzene. The reaction of 2-trifluoromethylphenoxazine with mixed chlorobromoalkanes in the presence of sodium amide in liquid ammonia gave N^{10}-(chloroalkyl)-2-trifluoromethylphenoxazine.

2-Trifluoromethylphenoxazine can be acylated by heating with chloroacetyl chloride in acetonitrile/ether medium to give N^{10}-chloroacetyl-2-trifluoromethylphenoxazine. Compounds (**2–21**) were prepared in good yield in two steps. The first step consisted of alkylating 2-trifluoromethylphenoxazine with 1-bromo-3-chloropropane or 1-bromo-4-chlorobutane to give 10-(3′-chloropropyl)-2-trifluoromethylphenoxazine (**2**) or 10-(4′-chlorobutyl)-2-trifluoromethylphenoxazine (**9**), alkylation being accomplished by first converting 2-trifluoromethylphenoxazine to the anionic species using a strong acid binding agent, sodium amide. Iodide catalysed nucleophilic substitution of the 10-propyl or butyl chloride of 2-trifluoromethylphenoxazine (**1**) with various secondary amines (N,N-diethylamine, N,N-diethanolamine, morpholine piperidine, pyrrolidine and β-hydroxyethylpiperazine) by refluxing for different times with potassium carbonate in acetonitrile gave the free bases (**3–8, 10–15**). The acetyl derivatives



Scheme 1

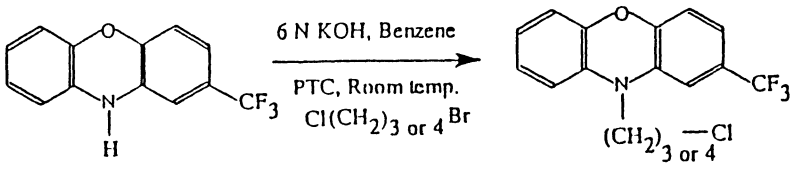
(**16–21**) were synthesised by the reaction of secondary amines under reflux conditions with 10-(chloroacetyl)-2-trifluoromethylphenoxazine (**16**) in acetonitrile containing potassium iodide.

Synthesis of N^{10}-chloropropyl or N^{10}-chlorobutyl derivatives of 2-trifluoromethylphenoxazine *via* phase transfer catalysis

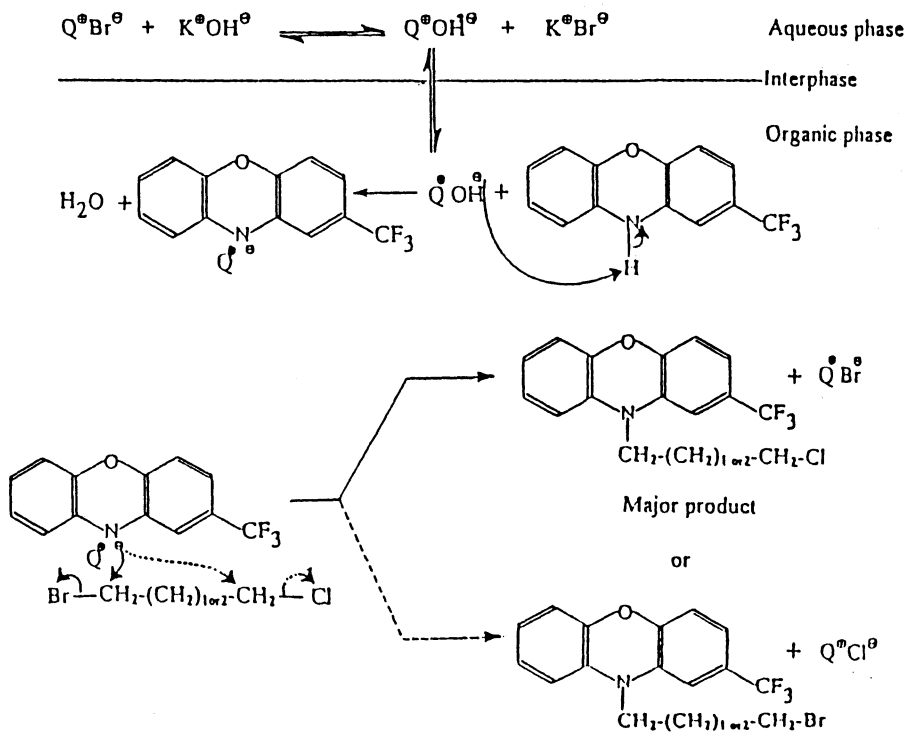
2-Trifluoromethylphenoxazine (**1**) has a less basic nitrogen atom and previously described preparative procedures for N-alkylation of this compound need sodamide in liquid ammonia or organometallic reagents. However, this compound undergoes N-alkylation in the presence of PTC more easily compared to previously described preparative procedures. Stirring of 2-trifluoromethylphenoxazine (**1**) at room temperature with alkylating agent (Br-$(CH_2)_3$-Cl or Br-$(CH_2)_4$-Cl) in a two phase system consisitng of an organic solvent (benzene) and a 6N aqueous potassium hydroxide solution in the presence of tetrabutylammonium bromide [$(n\text{-}C_4H_9)_4N^+Br^-$] leads to the formation of the compounds **2** or **9** in good yield. The reaction mechanism of the formation of the compounds in the presence of PTC is outlined in Scheme 2. Here, ammonium salt transports hydroxide ion from aqueous phase to organic phase where the actual reaction takes place. These results are interpreted by deprotonation of (**1**) by the OH^- ion, transferred by the catalyst into the organic layer. The anion formed may be regarded as phenolate stabilized anion, which subsequently undergoes alkylation to form the aromatized system. After checking the purity by HPLC, the compounds were characterized by UV, IR, ^1H and ^{13}C-NMR and mass spectral studies.

The UV spectra of the compounds revealed that each compound exhibits two bands (λ_{max}) at 223–245 nm and 319–335 nm, while the acetyl-2-trifluoromethylphenoxazines present a third additional absorption band at about 277–292 nm (aromatic ketone band). It has been found that N-alkylation has only a slight influence upon the position and intensity of these bands and merely produces small bathochromic shifts. The λ_{max} at 223–245 nm, 277–292 nm and 319–335 nm may be assigned respectively to $\pi \rightarrow \pi^*$, $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. Further, the UV-spectral data of 2-trifluoromethyl-N^{10}-substituted phenoxazines are in close agreement with the spectral characteristics of analogous heterocyclic compounds[29, 30].

The IR spectrum of 2-trifluoromethylphenoxazine reveals the characteristic absorption of *o*-substituted benzene rings and that of a secondary amino group. 2-trifluoromethylphenoxazine showed the N—H stretchng frequency at 3400 cm^{-1}. Bands in the 3390–3252 cm^{-1} region in the spectra of the compounds (**4, 8, 11, 15** and **21**) may be assigned to the O—H stretching frequency. The strong bands in the spectra between 3042 and 2400 cm^{-1} may be assigned to C—H stretching of the aromatic ring system. The bands observed at 1375–1250 cm^{-1} may be assigned for two C—N aromatic stretching vibrations. These signals in the range 1635–1400 cm^{-1} may be assigned to the ring stretching mode and the C—H out-of-plane bending modes in the region 880–645 cm^{-1}. The C—F stretching vibration was observed at 764–729 cm^{-1}. Similarly, bands in the region



Mechanism :



Where $Q^{\oplus} = (CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2)_4N^{\oplus}$

Scheme 2 N-Alkylation of 2-trifluromethylphenoxazine *via* phase transfer catalysis.

1230–1037 cm^{-1} may be assigned to the C—H in-plane-deformation vibrations in the heterocyclic aromatic six-membered ring system. The absorption bands in the range 1250–1150 cm^{-1} may be assigned to C—O diaryl stretching vibrations. Unsymmetrical trisubstituted derivatives have a band in the 800–744 cm^{-1} region. It has been shown that these bands are present in phenothiazines having a trifluoromethyl substituent[31, 32]. An examination of the IR spectra of the compounds dealing with the last region indicates the following ranges: hydrogen atoms in 1-, 2-, 3- and 4- positions, 765–735 cm^{-1}, hydrogen atoms in 1-, 2-, and 4- positios, 816–771 cm^{-1} (two adjacent hydrogen atoms) and 879–852 cm^{-1} (one

hydrogen atom). The IR bands indicated the presence of characteristic functional group and peaks at 1691–1626 cm^{-1} indicated the presence of >C=O group in the acetyl derivatives. Thus, the characteristic bands in the IR spectra support the structures of phenoxazines.

The ^{1}H-NMR spectrum, typical of phenoxazine compound, showed seven aromatic protons (multiplet) at 'δ' 6.42–7.29 (m, H_1, H_3, H_4, H_6-H_9) for propyl and butyl derivaitves (**1–15**) and at 'δ' 7.25–8.12 (m, H_1, H_3, H_4, H_6-H_9) for acetylphenoxzines (**16–21**). A triplet at 'δ' 1.37 was assigned to methyl protons of the compound **3** and quartet at 'δ' 3.24 to methylene protons (H_a and H_b) with $J \approx 8$ Hz. In the proton spectrum of **4**, a singlet at 'δ' 3.04 assigned to the protons (H_e and H_f) of —OH groups which disappeared on D_2O exchange. In the spectrum of **5**, a triplet at 'δ' 2.41 was exhibited by the protons H_a and H_b with a coupling constant $J \approx 13.2$ Hz, whereas in the spectrum of **7** signal at 'δ' 1.75 was assigned to the six protons H_1, H_c and H_d with a coupling constant $J \approx 12.1$ Hz. The proton H_g which exhibited the singlet at 'δ' 3.25 in the case of **8** disappeared on D_2O exchange. In the spectrum of **15** the signal at 'δ' 3.24 due to H_g of the —OH group disappeared on D_2O exchange and a triplet at 'δ' 3.44 due to H_f protons showed a coupling constant $J \approx 7$ Hz. Signals at 'δ' 2.33–2.49 was assigned to twelve protons (H_a, H_b, H_c, H_d, H_n and H_e). In the spectrum of **17** a signal at 'δ' 1.17 was a triplet due to the protons H_c and H_d with a coupling constant $J \approx 8$ Hz. Comparison of the ^{1}H-NMR spectra of 2-trifluoromethylphenoxazines and those of corresponding N^{10}-substituted 2-trifluoromethylphenoxazines showed clearly that the only difference between the spectra of each N^{10}-unsubstituted and N^{10}-substituted pair was the bulk chemical shift of all common hydrogen atoms. The individual chemical shifts of each aromatic hydrogen atom and the coupling constants were the same. A combination of chemical shift, spin-spin couplings and integration data permits the identification of individual hydrogens at each side in the aromatic ring. The assignment of protons in the case of twentyone compounds (**1–21**) is fully supported by the integration curves and all the derivatives showed the characteristic chemical shifts for the 2-trifluoromethylphenoxazine nucleus.

The ^{13}C-NMR spectrum of each 2-trifluoromethyl-N^{10}-substituted phenoxazines exhibited twelve signals representing twelve aromatic carbons. The twelve aromatic carbon atoms showed the signals at 'δ' (^{1}H-decoupled): 107.76 (C_1), 124.29 (C_2), 118.52 (C_3), 115.73 (C_4), 115.30 (C_6), 121.72 (C_7), 124.30 (C_8), 115.52 (C_9), 133.56 ($C_{1'}$), 144.24 ($C_{4'}$), 144.4 ($C_{6'}$), 133.56 ($C_{9'}$) for alkyl derivatives and 117.34 (C_1), 124.52 (C_2), 123.70 (C_3), 124.64 (C_4), 122.50 (C_6), 124.64 (C_7), 127.9 (C_8), 117.50 (C_9), 128.11 ($C_{1'}$), 150.37 ($C_{4'}$), 154.00 ($C_{6'}$) and 127.00 ($C_{9'}$) for acetyl derivatives. The resonances at 'δ' 144.24 and 144.34 ppm were assigned to the bridged head carbons $C_{4'}$ and $C_{6'}$ respectively. The chemical shift at the lower field was assigned to the carbon adjacent to oxygen probably due to a larger deshielding effect of carbon resonances and higher electronegativity of oxygen. Similarly, the chemical shifts at 'δ' 107.76 and 111.52 ppm were assigned to the carbons *ortho* (C_1 and C_9) and at 'δ' 118.52 and 121.72 ppm to the carbons were assigned to *para* (C_3 and C_7) to nitrogen. The remaining resonances at 'δ' 115.73 and 115.30 ppm to carbons C_4 and C_6 and at

'δ' 124.29 and 124.30 to carbons C_2 and C_8 were assigned. The assignments of the ^{13}C-resonances of 2-trifluoromethylphenoxazines are in close agreement with analogous compounds, 2-chlorophenothiazines[33]. In toto, ^1H- and ^{13}C-NMR spectral data were consistent with the proposed structures for 2-trifluoromethyl-N^{10}-substituted phenoxazines.

Recently, a set of eight 2-trifluoromethyl-N^{10}-subsituted phenoxazines were characterized by using electron ionization and liquid secondary ionization mass spectrometric techniques[34]. To provide a visual picture, the mass-spectrum of compound (**3**) is illustrated in Fig. 1. A closer look at the mass spectral data reveals that at 2-trifluoromethyl phenoxazines yield abundant molecular ions. The molecular ion is the base peak in the mass spectra of these compounds except in the spectra of **2, 4, 9, 12, 16** and **19**; the relative abundances of the molecular ions of these compounds lie in the range 30–81%. In general, mass spectral features of 2-trifluoromethylphenoxazines are similar. The phenoxazine ring system remains stable, whereas fragmentation reactions are observed due to cleavage of bonds in the N^{10}-side chain portion of these compounds. A similar observation has been reported for the phenothiazine derivatives[35–37].

The effectiveness of any agent as an MDR modulator will depend in part on its ability to accumulate in cells. 2-Trifluoromethylphenoxazines are weak bases and able to exist in both charged (protonated) and uncharged (unprotonated) forms. The unprotonated or neutral form of compounds will be highly membrane permeable and able to diffuse freely and rapidly across biological membranes. In contrast, the protonated form would be at least an order of magnitude less membrane permeable and diffuse across membranes at a much reduced rate[38]. In addition, if the unprotonated form of the molecule diffuses across the membrane and enters an acidic compartment within the cell, it will rapidly become protonated and unable to diffuse out of the cell. The magnitude of the biological activity depends on pKa of compounds besides other factors. For the series of compounds examined, the pKa values (Table-1) ranging from 7.17–9.45 lie closer to physiological pH, which may sugest that these compounds accumulate in MDR cells as free bases than in protonated form. The lipophilicity data varying from 1.40–2.60 expressed in $\log_{10} P$ for twentyone 2-trifluoromethyl-N^{10}-substituted phenoxazine derivatives (**1–21**) are given in Table-1. Within this series, all compounds are highly lipophilic at pH 7.4 and it is expected that they will accumulate rapidly into cells.

Evaluation of 2-trifluoromethyl-N^{10}-substituted phenoxazines for anti-MDR activity

In an attempt to determine the structural requirements of the modulators for better anti-MDR activity, previously Thimmaiah *et al.*[23] have found that parameters such as lipophilicity, a tricyclic ring with an —NH group at position 10 and a highly electronegative atom like oxygen at position 5 seem to be essential. Subsequently, twenty N^{10}-substituted phenoxazines[26, 39] were examined for their anti-MDR activity and within the series there are compounds that inhibit efflux (VRP-like activity), whereas others markedly increase vinca alkaloid

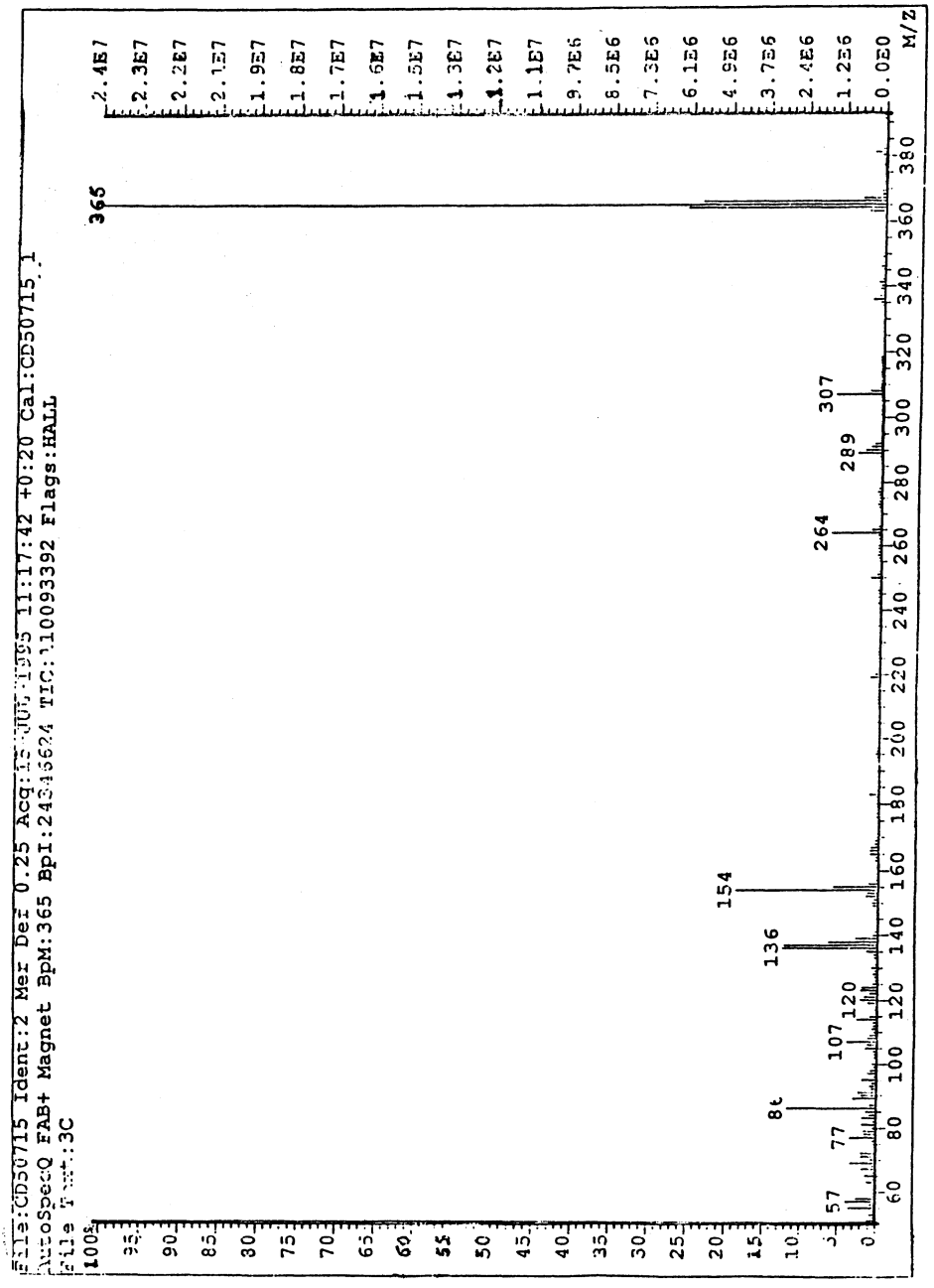


Fig. 1 Mass spectrum of 10[3'-(N-diethylamino)propyl]-2-trifluoromethyl phenoxazine

TABLE-1
EFFECT OF 2-TRIFLUOROMETHYL-N^{10}-SUBSTITUTED PHENOXAZINES ON THE ACCUMULATION OF VINBLASTINE IN $KBCH^R$-8-5 CELLS

Compound No.	pKa	$\log_{10} P^a$	Vinblastine uptake[b] (% control)
1	7.57	2.10	640
2	7.67	1.80	320
3	4.84 8.50	1.70	163ε
4	5.12 9.35	2.60	5850
5	4.47 8.90	2.60	2290
6	3.60 8.30	1.70	1524
7	4.25 8.30	1.80	1117
8	4.45 8.30	1.46	1512
9	7.65	2.00	160
10	5.25 8.60	1.58	984
11	4.25 8.55	1.60	ND
12	4.75 8.97	2.00	3840
13	3.55 8.05	1.60	828
14	4.65 8.90	1.52	950
15	5.25 9.45	1.80	3000
16	7.17	1.40	680
17	4.73 8.70	1.50	557
18	4.20 8.10	1.58	425
19	4.87 9.30	1.60	1090
20	5.15 8.95	1.65	325
21	5.15 8.55	1.70	533
VRP			748

[a]Octanol/buffer partition coefficient

[b]$\left(\frac{\text{VLB uptake with modulator}}{\text{VLB uptake without modulator}}\right) \times 100$

Compoounds were tested at 100 μM. Each experiment was done in triplicate.

accumulation without having detectable inhibitory activity on the efflux component. Additionally, certain of these modulators significantly enhance accumulation of vinca alkaloids in cell lines with very low or undetectable P-gp levels, where VRP has little activity, suggesting that N^{10}-substituted phenoxazines demonstrate both quantitative and qualitative differences compared with VRP. Based on the uptake and efflux data and competition for azidopine binding to P-gp, it was tentatively concluded that at least part of the activity of N^{10}-substituted modulators may be mediated through a P-gp independent mechanism[39].

Effect of 2-trifluoromethyl N^{10}-substituted phenoxazines on the accumulation of vinbalstine in MDR cells

The effect of twentyone modulators at 100 μM on the steady state accumulation of vinblastine (VLB) in drug resistant $KBCh^R$-8-5 cells was determined and data are given in Table-1. As shown in Table-1, **3–8, 10** and **12–15** at 100 μM concentration exhibited a significant VLB accumulation enhancing effect (8.30 to 58.50-fold relative to control) compared to a standard modulator, VRP (7.5-fold) and caused a 1.10 to 7.82-fold greater accmulation of VLB than did a similar concentration of VRP. A similar effect was observed in the effect of twentyone 2-chlorophenoxazines on the accumulation of VLB in drug resistant $KBCh^R$-8-5 cells[40]. Further, the effect of the varying concentration of compounds **1, 2, 4, 5, 9, 12, 16** and **19** or VRP on the uptake of VLB in MDR cells was studied. The results are given in Fig. 2 (**2, 9, 16** or **VRP**) and figure 3 (1, 4, 5, 12, 19 or VRP). The data revealed that all the modulators at 10 μM exhibited the

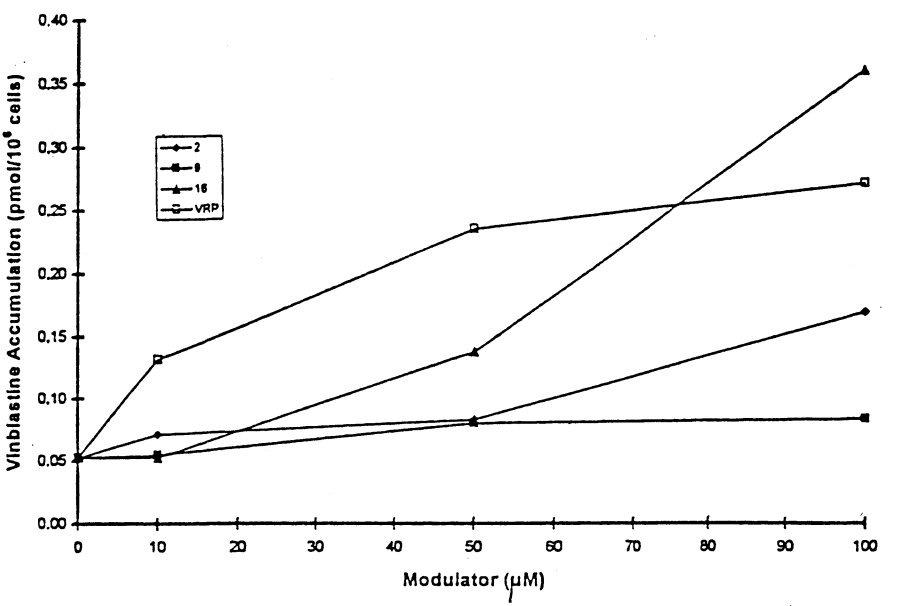


Fig. 2 Accumulation of [^{3}H]vinblastine 25nM) in $KBCH^R$-8-5 cells with increasing concentrations of 2-trifluoromethyl phenoxazines or verapamil. Compound **2** (◆), compound **9** (■), compound **16** (▲) and verapamil (□).

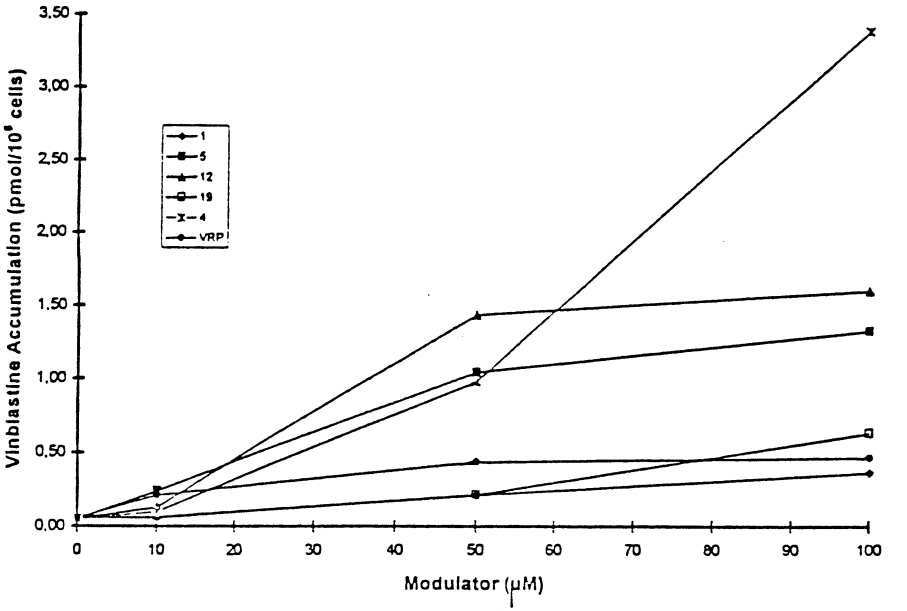


Fig. 3 Accumulation of [^{3}H]vinblastine 25nM) in KBCH^R-8-5 cells with increasing concentrations of 2-trifluoromethyl phenoxazines or verapamil. Compound **1** (◆), compound **5** (■), compound **12** (▲) and verapamil **19** (□), compound **4** (*) and verapamil (•).

least VLB enhancing effect whereas at 50 μM and 100 μM, the modulators exhibited a significant increase in the accumulation of VLB suggesting that the uptake of vinblastine into KBCh^R-8-5 cells was dependent on the concentration of the modulator. The VLB accumulation enhancing effects with respect to control (%) are in the range 320–5850 for propyl derivatives, 160–3840 for butyl derivatives and 325–1090 for acetyl derivatives, suggesting that the propyl derivatives seem to rank in the list of these compounds. The three compounds substituted at N^{10}-with only chlorine attached to the propyl, butyl or acetyl (**2, 9** and **16**) were found to be marginally effective at increasing VLB accumulation in MDR KBCh^R-8-5 cells. However, no suitable explanation was offered for the compound **16** with VLB accumulation enhancing effect of 680% being greater than the compounds **2** and **9**.

The presence of N^{10}-substitution seems to be necessary to optimise activity in the 2-trifluoromethyl phenoxazines. Comparison of the derivatives of their ability to potentiate uptake of VLB in KBCh^R-8-5 cells revealed that they largely follow the order: **N^{10}-propyl > N^{10}-butyl > N^{10}-acetyl** derivatives. Modulators (**16–21**) exhibited the least VLB uptake enhancing effect in MDR KBCh^R-8-5 cells probably because the attachment of polar group —$COCH_2$— to the N^{10}-position confers increased hydrophilicity to the molecule.

In order to elucidate the role played by —CF_3 group in position C-2 of the phenoxazine ring the accumulation data of the present series of compounds (**1–21**) was compared with those of the corresponding phenoxazine derivatives where

position C-2 of the ring is occupied by a hydrogen atom[26]. In general, substitution of hydrogen by —CF_3 in position C-2 increased the efficacy to enhance the uptake of VLB in KBCh^R-8-5 cells by 1.1 to 6.3-fold except **2, 9, 13, 17–21**. Additionally, it is speculated that the phenoxazine nucleus with —CF_3 at position C-2 may exhibit a higher affinity for membranes or be more readily taken up into cells than that with a hydrogen atom, an observation made previously in analogous phenothiazine compounds.

Effect of substituted phenoxazines on the efflux of [^{3}H] VLB from MDR cells

Steady state accumulation of VLB, a substrate for P-glycoprotein mediated efflux[40] was studied in the MDR cell line KBCh^R-8-5 in the presence and absence of novel MDR modulators. To determine whether the increase in VLB accumulation upon co-incubation with 2-trifluoromethylphenoxazines was due to a slowing of P-glycoprotein mediated VLB efflux, KBCh^R-8-5 cells were loaded with 55.6 nm [^{3}H] VLB in the presence of modulator (100 μM), then incubated in VLB free medium containing the same modulator. The cell associated radio-label remaining after a 2 h efflux was determined and calculated.

When KBCh^R-8-5 cells were loaded with VLB in the absence of modulator, less than 10% of the cellular VLB remained after 2 h. When a similar experiment was performed, but with 100 μM concentration of **8** or VRP present continuously, about 40% of the cell-associated VLB remained at the end of a 2 h efflux period (Fig. 4). These results suggest that 2-trifluoromethyl phenoxazines, like VRP, are able to inhibit P-glycoprotein mediated VLB efflux from KBCh^R-8-5 cells.

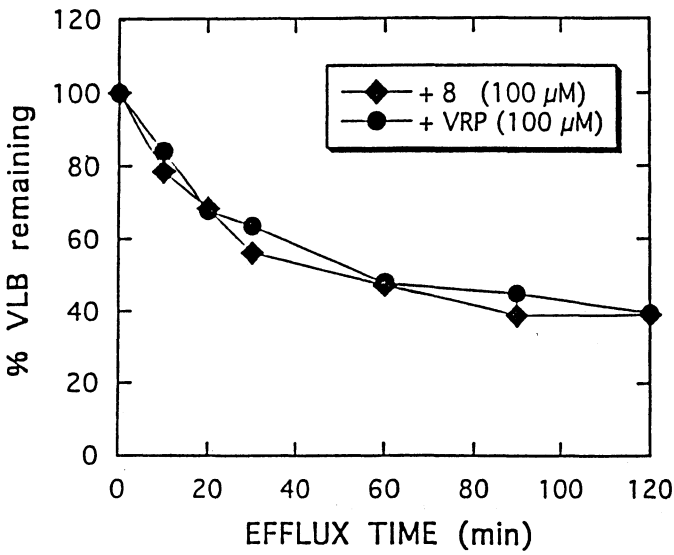


Fig. 4 Effect of modulator **8** and verapamil on the outward transport of [^{3}H]vinblastine from KBCH^R-8-5 cells.

Inhibition of clonogenic potential by phenoxazine MDR modulators

The cytotoxicity of each of the twentyone 2-trifluoromethyl-N^{10}-substituted phenoxazines (**1–21**) was examined in MDR $KBCh^R$-8-5 cells. The percent survival curves for the compounds **1–8, 9–15** and **16–21** in the case of $KBCh^R$-8-5 cells are given respectively in Figures 5, 6 and 7. The concentration of modulators (**1–21**)

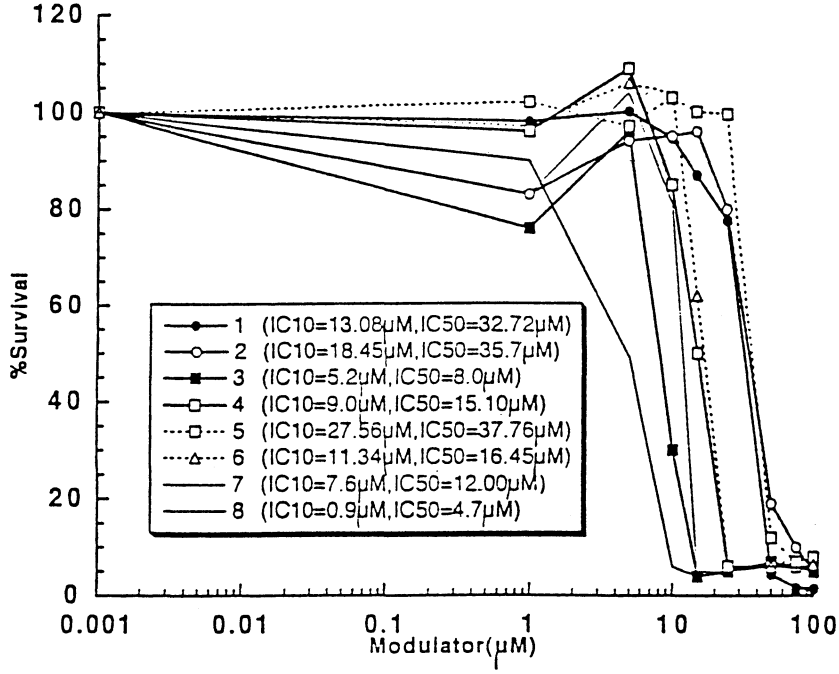


Fig. 5 Cytotoxicity of 2-trifluoromethyl phenoxazine derivatives (**1–8**) in drug resistant $KBCH^R$-8-5 cells.

that reduced colony formation by 10% and 50% (IC_{10} and IC_{50}) were determined from the concentration per cent survival curves and the results are given in Table-2. The IC_{10} and IC_{50} values for twentyone modulators (**1–21**) lie in the range of 0.10 to 39.90 μM and 2.10 to 70.90 μM respectively for $KBCh^R$-8-5 cells.

Screening of six 2-trifluoromethylphenoxazines for their anticancer activity against different tumor cell lines

The cytocidal activity of six 2-trifluoromethyl phenoxazines (**3, 5, 8, 11, 13** and **15**) was determined against eight cancer cell lines [colon (SW-620), CNS (U251), Ovarian (SKOV-3), breast (MCF-7/ADR), Prostate (DU 145), renal (A 498), Lung (H 522) and melanoma (M 14)]. The tumor cells were exposed to varying concentrations of each phenoxazine and IC_{50} values were determined and the results are given in Table-3. The anti-proliferaive activity of these compounds was compared to the standard drug, topotecan. The data revealed that compound **15** was found to be more potent than topotecan against colon SW-620 and breast MCF-7/ADR cancer cells and compounds **3, 5** and **8** showed significant activity against ovarian cancer cells.

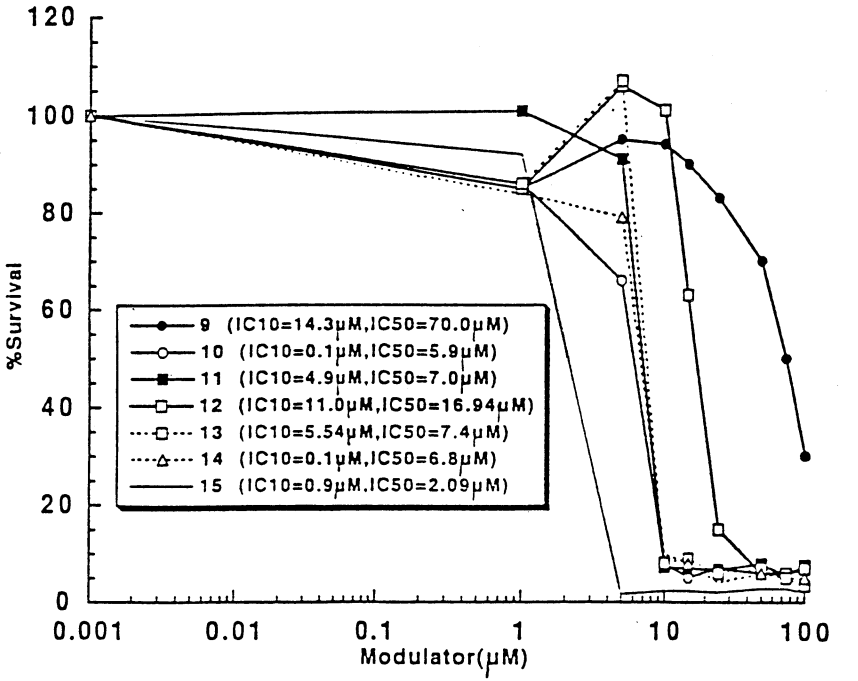


Fig. 6 Cytotoxicity of 2-trifluoromethyl phenoxazine drivatives (**9–15**) in drug resistant $KBCH^R$-8-5 cells.

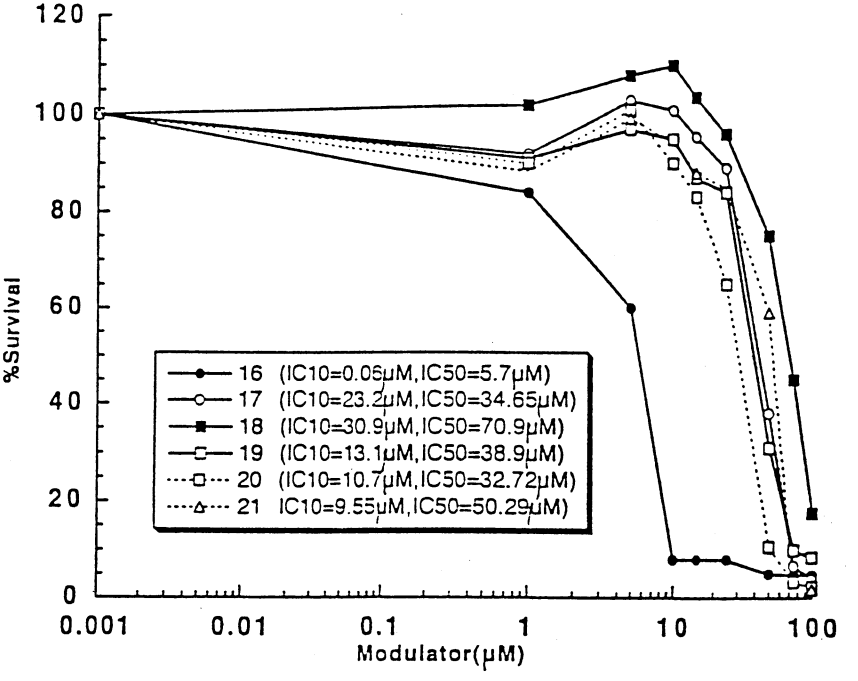


Fig. 7 Cytotoxicity of 2-trifluoromethyl phenoxazine drivatives (**16–21**) in drug resistant $KBCH^R$-8-5 cells.

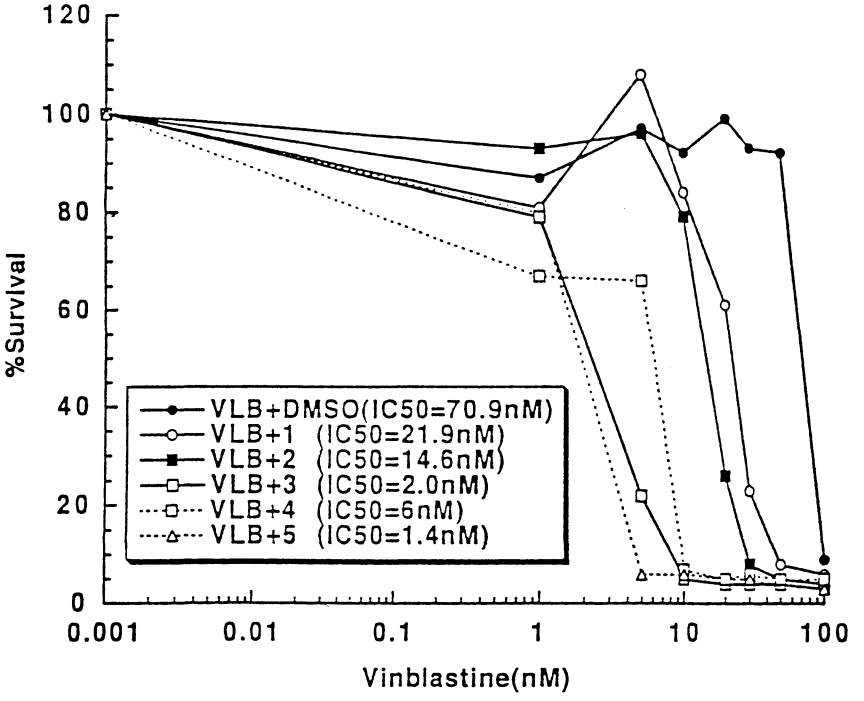


Fig. 8 Effect of 2-trifluoromethyl phenoxazine modulators (**1–5**) on the cytotoxicity of vinblastine in KBCH^R-8-5 cells.

TABLE-2
CYTOTOXICITY OF 2-TRIFLUOROMETHYL-N^{10}-SUBSTITUTED PHENOXAZINES IN HUMAN KBCH^R-8-5 CELLS

Compund no.	IC_{10} (μM)	IC_{50} (μM)	Compund no.	IC_{10} (μM)	IC_{50} (μM)
1	13.08	32.72	12	11.00	16.94
2	18.45	35.70	13	5.54	7.40
3	5.20	8.00	14	0.10	6.80
4	9.00	15.10	15	0.90	2.09
5	27.56	37.76	16	0.06	5.70
6	11.34	16.45	17	23.20	34.65
7	7.60	12.00	18	30.90	70.90
8	0.90	4.70	19	13.10	38.90
9	14.30	70.00	20	10.70	32.72
10	0.10	5.90	21	9.55	50.29
11	4.90	7.00			

IC_{10} and IC_{50} are the concentrations (μM) required to produce 10% and 50% reduction respectively, in clonogenic survival of cells under the conditions described in the experimental section.

TABLE-3
ANTICANCER ACTIVITY OF SIX 2-TRIFLUOROMETHYL-N^{10}-SUBSTITUTED PHENOXAZINES AGAINST DIFFERENT TUMOR CELL LINES

Cell line	Compound number and IC_{50} values						
	3	5	8	11	13	15	Topotecan (Standard drug)
Colon (SW-620)	2×10^{-5}	8×10^{-5}	3×10^{-5}	6×10^{-6}	2×10^{-5}	$<10^{-8}$	9×10^{-7}
CNS (U251)	4.5×10^{-5}	$>10^{-4}$	3×10^{-5}	2×10^{-5}	1×10^{-5}	$>10^{-4}$	6×10^{-8}
Ovarian (SKOV-3)	2.5×10^{-5}	9×10^{-7}	1×10^{-8}	5×10^{-6}	3×10^{-5}	5×10^{-6}	4×10^{-7}
Breast (MCF7/ADR)	3×10^{-5}	5.5×10^{-5}	1×10^{-5}	2×10^{-5}	2×10^{-5}	$<10^{-8}$	9×10^{-8}
Prostate (DU 145)	2.5×10^{-5}	5×10^{-5}	1×10^{-6}	3×10^{-5}	2×10^{-5}	9×10^{-7}	5×10^{-8}
Renal (A498)	2.5×10^{-5}	9×10^{-5}	4×10^{-7}	3×10^{-6}	6×10^{-7}	1×10^{-7}	3×10^{-7}
Lung (H522)	3×10^{-5}	$>10^{-4}$	1×10^{-5}	2×10^{-5}	2×10^{-5}	1×10^{-5}	6×10^{-7}
Melanoma (M14)	5×10^{-5}	$>10^{-4}$	4×10^{-6}	5×10^{-6}	8×10^{-6}	5×10^{-6}	3.5×10^{-7}

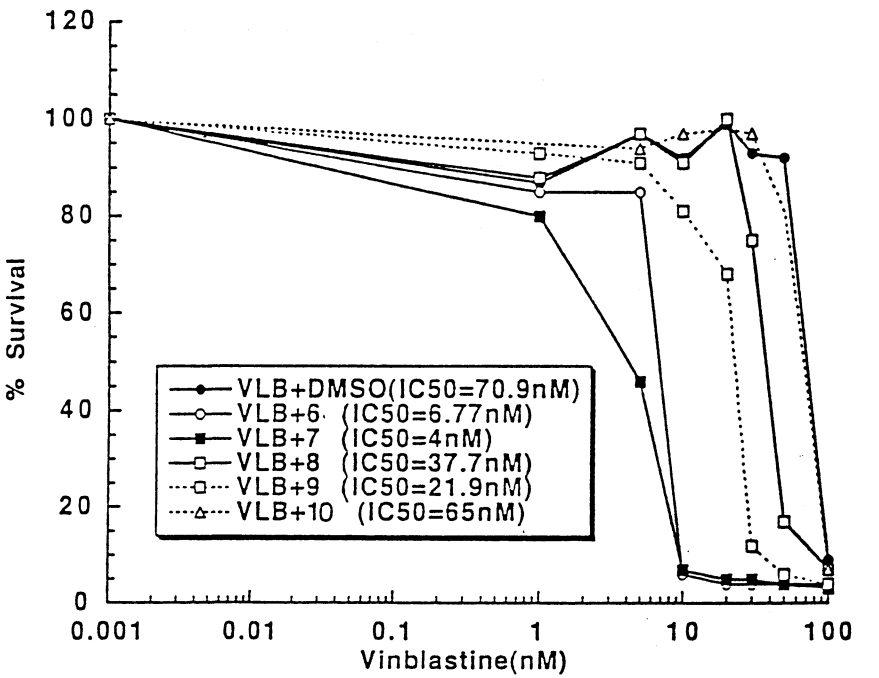


Fig. 9 Cytotoxicity of 2-trifluoromethyl phenoxazine drivatives (**6–10**) in drug resistant $KBCH^R$-8-5 cells.

Effect of 2-trifluoromethyl-N^{10}-substituted phenoxazines on *in vitro* cytotoxicity of VLB

We have evaluated the ability of twentyone compounds (**1–21**) to modulate the cytotoxicity of VLB in MDR cells. Cells were exposed continuously for seven days to 0–100 nM VLB in the absence and presence of IC_{10} concentrations of 2-trifluoromethyl phenoxazine modulators. The concentration response curves [Figures 8, 9, 10 and 11] were determined by clonogenic assay, and the IC_{50} and fold-potentiation of VLB cytotoxicity are summarized in Table-4. The modulators tested at the IC_{10} enhanced the cytotoxicity of VLB by 1.03 to 51-fold against $KBCh^R$-8-5 cells. Examination of the cytotoxicity data of VLB in the presence of compounds **1–21** revealed that the modulator **5** demonstrated the greatest effect followed by **3, 11, 13, 7, 18, 4, 6, 12, 19** and so on. Only four compounds (**3, 5, 11** and **13**), like VRP, were able to completely reverse the 24-fold resistance of $KBCh^R$-8-5 cells to VLB. The IC_{50} value for continuous exposure to VLB was 3.0 nM in KB-3-1 and 70.9 nm in $KBCh^R$-8-5 cells in the absence of modulators. The most effective agents in $KBCh^R$-8-5 cells were subsequently tested in KB-3-1 and all were shown to cause a small sensitization (3.0–3.8-fold) of this drug senstivie line to VLB. However, a similar degree of sensitization was achieved when used the classical MDR modulator, VRP (3.8-fold).

TABLE-4
EFFECT OF 2-TRIFLUOROMETHYL-N^{10}-SUBSTITUTED PHENOXAZINES ON THE POTENTIATION OF VINBLASTINE CYTOTOXICITY IN DRUG RESISTANT $KBCH^R$-8-5 CELLS

Compound no.[a]	Vinblastine IC_{50} (μM)	Fold potentiation	Compound no.[a]	Vinblastine IC_{50} (μM)	Fold potentiation
1	21.90	3.00	12	6.70	Complete
2	14.60	5.00	13	2.00	Complete
3	2.00	Complete[b]	14	43.50	1.50
4	6.00	Complete	15	41.00	2.00
5	1.40	Complete	16	68.90	1.03
6	6.70	Complete	17	7.80	9.00
7	4.00	Complete	18	4.80	Complete
8	37.70	2.00	19	7.00	Complete
9	21.90	3.00	20	22.50	3.00
10	65.00	1.10	21	22.50	3.00
11	2.00	Complete			

[a]Modulators used at the IC_{10} concentration (Table-2)
[b]Complete reversal of vinblastine resistance

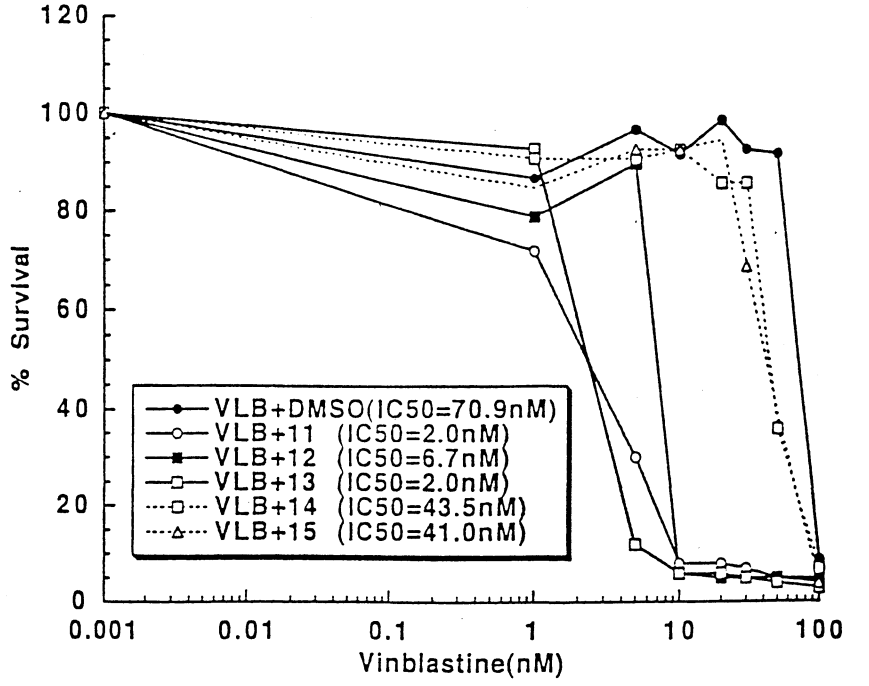


Fig. 10 Cytotoxicity of 2-trifluoromethyl phenoxazine drivatives (**11–15**) in drug resistant $KBCH^R$-8-5 cells.

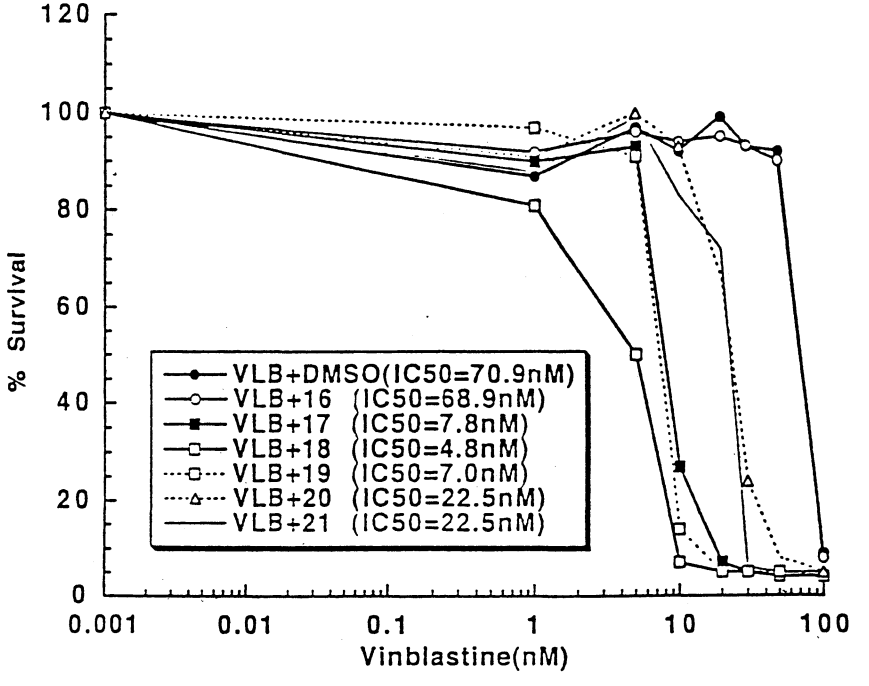


Fig. 11 Cytotoxicity of 2-trifluoromethyl phenoxazine drivatives (**16–21**) in drug resistant $KBCH^R$-8-5 cells.

REFERENCES

1. R.L. Juliano and V.A. Ling, *Biochem. Biophys. Acta.*, **455**, 152 (1976).
2. I.B. Roninson, H. Abelson, D.E. Housman, N. Howell and A. Varshavsky, *Nature*, **309**, 626 (1984).
3. K. Ueda, M.M. Cornwell, M.M. Gottesman, I. Pastan, I.B. Roninon, V. Ling and J.R. Riordan, *Biochem. Biophys. Res. Commun.*, **141**, 956 (1986).
4. M. Hario, M.M. Gottesman and I. Pastan, *Proc. Natl. Acad. Sci. (USA)*, **85**, 3580 (1988).
5. L.J. Goldstein, M.M. Gottesman and I. Pastan, *Cancer Treat. Res.*, **57**, 101 (1991).
6. L.J. Goldstein, I. Pastan and M.M. Gottesman, *Crit. Rev. Oncol. Hematol.*, **12**, 243 (1992).
7. S.E. Bates, Y.S. Ching and M. Tsokos, *Am. J. Pathol.*, **139**, 305 (1991).
8. P. Borst, *Rev. Oncol.*, **4**, 87 (1991).
9. T. Tsuruo, H. Iida, S. Tsukagoshi and Y. Sukurai, *Cancer Res.*, **41**, 1967 (1981).
10. R. Ganapathi and D. Grabowski, *Cancer Res.*, **43**, 3696 (1983).
11. T. Tsuruo, H. Iida, Y. Kitatani, K. Yokota, S. Tsukgoshi and Y. Sakurai, *Cancer Res.*, **44**, 4303 (1984).
12. J.M. Zamora and W.T. Beck, *Biochem. Pharmacol.*, **35**, 4303 (1986).
13. J.M. Zamora, H.L. Pearce and W.T. Beck, *Mol. Pharmacol.*, **33**, 454 (1988).
14. C.H. Yong, S.G. DePinho, L.M. Greenberger, R.J. Arceci and S.B. Horowitz, *J. Biol. Chem.*, **264**, 782 (1989).
15. A. Ramu, D. Glaubiger and Z. Fuks, *Cancer Res.*, **44**, 4392 (1984).
16. L.M. Slater, P. Sweet, M. Stupecky and S. Gupta, *J. Clin. Invest.*, **77**, 1405 (1986).
17. Y. Keisuke and T. Tsuruo, *Cancer Res.*, **49**, 5002 (1989).
18. X. Qian and W.T. Beck, *Cancer Res.*, **50**, 1132 (1990).
19. R.F. Ozols, R.E. Cunnion, R.W. Klecker, T.C. Hamilton, Y. Ostchega, J.E. Parrillo and R.C. Young, *J. Clin. Oncol.*, **5**, 641 (1987).
20. W.S. Dalton, T.M. Grogan, P.S. Meltzer, R.J. Scheper, B.G. Durie, C.W. Taylor, T.P. Miller and S.E. Salmon, *J. Clin. Oncol.*, **7**, 415 (1989).
21. T. Fojo, N. McAtee, C. Allegra, S. Bates, L. Mickley, H. Keister, M. Linehan, S. Stienberg, E. Tucker and L. Goldstein, *Proc. Am. Soc. Clin. Oncol.*, **8**, A262 (1989).
22. J.M. Ford and W.N. Hait, *Pharm Rev.*, **42**, 155 (1990).
23. K.N. Thimmaiah, J.K. Horton, Xiao-dong Qian, W.T. Beck, J.A. Houghton and P.J. Houghton, *Cancer Commun.*, **2**, 249 (1990).
24. K.A.M. Walker, D.R. Hirschfeld and M. Marx, *J. Med. Chem.*, **21**, 1335 (1978).
25. W.B. Butler, *Analyt. Biochem.*, **141**, 70 (1984).
26. K.N. Thimmaiah, J.K. Horton, R. Seshadri, M. Israel, J.A. Hougtton, F.C. Harwood and P.J. Houghton, *J. Med. Chem.*, **35**, 3358 (1992).
27. N. Motohashi, L.A. Mitscher and R. Meyer, *Med. Res. Rev. II*, **239** (1991).
28. H. Fuhrer, P. Sutter and C.D. Weis, *J. Heterocyclic Chem.*, **16**, 1121 (1979).
29. A. Butenandt, E. Biekert and W. Schafer, *Ann. Chem.*, **632**, 134 (1960).
30. M. Ogata and H. Kano, *Tetrahedron*, **24**, 3725 (1968).
31. N.L. Smith, *J. Org. Chem.*, **15**, 1125 (1950).
32. A. Roe and W.F. Little, *J. Org. Chem.*, **20**, 1577 (1953).
33. M.V. Jovanovic and R. Edward Biehl, *Organic Magnetic Resonance*, **22**, 49 (1994).
34. C. Das, K.N. Thimmaiah, B.S. Jayashree, R. Sheshadri, M. Israel and P.J. Houghton, *Bio. Mass. Spectrum.*, **23**, 140 (1994).
35. A.M. Duffied, J. Cymerman Craig and L.R. Kray, *Tetrahedron*, **24**, 4267 (1968).
36. S. Morosawa, S. Kamal, P.C. Dandiya and H.L. Sharma, *Org. Mass Spectrom.*, **17**, 309 (1982).
37. A. Kumar, J.N. Sinha and K. Shankar, *Org. Mass Spectrom.*, **18**, 497 (1983).
38. C. Deduve, T. Debrasy, B. Poole, A. Trouct, P. Tukens and F. Vanttof, *Biochem. Pharmacol.*, **23**, 2495 (1974).
39. J.K. Horton, K.N. Thimmaiah, F.C. Harwood, J.F. Kuttesch and P.J. Houghton, *Mol. Pharmacol.*, **44**, 552 (1993).
40. M. Hario, M.M. Gottesmann and I. Pastan, *Proc. Natl. Acad. Sci. (USA)*, **86**, 5128 (1989).

(*Received*: 14 January 1999; *Accepted*: 3 April 1999) AJC-1713