RESEARCH PAPER

Accepted 10 April 1999
Received 10 October 1998
Indian J. Pharm Sci. 1999, 61 (3) 168-174

Effect of Phenoxazine MDR Modulators on Photoaffinity Labeling of P-glycoprotein by [^{3}H] Azidopine: An Approach to Understand Drug Resistance in Cancer Chemotherapy

H.N. KALPANA, G.B. EREGOWDA, S. JAGADEESH AND K.N. THIMMAIAH*
Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore-570 006

Previously, a series of 21 N^{10}-substituted phenoxazines were examined for reversing vinca alkaloid resistance against MDR KBCh^R-8-5 and GC_3/cl cells. Within the series, there are compounds that inhibit efflux (verapamil-like activity), whereas others markedly increased vinca alkaloid accumulation without having detectable inhibitory activity of the efflux component. It has been shown that MDR modulators that inhibit photoaffinity labeling of P-gp were generally the most potent MDR reversers. To show whether this observation is true, P-gp rich membrane fractions from KB-V1 cells were isolated and the interaction of [^{3}H] azidopine with membrane fractions in the presence of 25, 50 and 100 μM concentration of each of the twenty N^{10} -substituted phenoxazines was undertaken and the extent of competition was compared to a standard modulator, verapamil. Examination of the competition data showed that only two modulators 4 and 6 exhibited the maximum competition (>50%) and the remaining modulators were found to exhibit the inhibition of the photolabeling by less than 45%. However, modulators 12, 14 and 19 failed to compete for azidopine labeling. Within the series of compounds examined, the competition of phenoxazines for [^{3}H] azidopine binding to P-gp follows the order: propyl>butyl>acetyl series. It has been found that, from among the compounds examined, three of them interact strongly (>50%), six marginally (<45%) and remaining failed to interact with P-gp, indicating that there may be multiple mechanisms for MDR.

Multidrug resistance (MDR) has been recognised as a major obstacle in the clinical treatment of cancer. The exposure of malignant cells to natural cytotoxic drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxines, colchicine, actinomycin D, taxol and some other compounds, frequently results in the emergence of cell populations resistant to the selecting agent as well as to other mechanistically distinct and structurally unrelated compounds[1] and this phenomenon is called multidrug resistance (MDR). This MDR phenotype is due in most cases, to a reduction in the intracellular accumulation of these drugs by resistant cells and enhanced efflux, which is believed to be a result of increased expression of a 170 kDa transmembrane glycoprotein known as P-glycoprotein (P-gp) encoded by the mdr 1 gene in human cells[2,3]. P-gp functions as an energy-dependent efflux pump which transports cytotoxic drugs out across plasma membrane, resulting in a decrease of drug accumulation to sublethal levels within the cell. The amount of P-gp was found to correlate with both the degree of resistance and the relative decrease in drug accumulation[4], but such a correlation is not always found[5].

MDR has been shown to be circumvented *in vitro* by a large number of compounds which have been grouped into ten broad categories: (a) calcium channel blockers[6], (b) calmodulin antagonists[7], (c) non-cytotoxic anthracycline and vinca alkaloid analogs[8], (d) steroid and hormonal analogs[9], (e) antiarrhythmics[10,11] (f) antimalarials[12], (g) lysosomotropic agents[13], (h) antiestrogens[14], (i) cyclosporins[15] and (j) cyclic peptide antibiotics[16]. They have been found to lower the IC_{50} of a

*For Correspondence

Table I - Competition by N^{10}-Substituted Phenoxazines for [^{3}H] Azidopine Binding

Modulator	Name	[^{3}H] Azidopine binding (% control)
1	10-(3'-Chloropropyl) phenoxazine	55
2	10-[3'-(N-Diethylamino)propyl]phenoxazine	70
3	10-[3'-[N-Bis(hydroxyethyl)amino]propyl] phenoxazine	98
4	10-(3'-N-Morpholinopropyl)phenoxazine	50
5	10-(3'-N-Piperidinopropyl)phenoxazine	71
6	10-[3'-[(β-Hydroxyethyl)piperazino]propyl]phenoxazine	36
9	10-[4'-(N-Diethylamino)butyl]phenoxazine	96
10	10-[4'-(N-Bis(hydroxyethyl)amino[buty]phenoxazine	76
11	10-[4'-N-Morpholinobutyl)phenoxazine	55
12	10-(4'-N-Piperidinobutyl)phenoxazine	103
13	10-[4'-[(β-Hydroxyethyl)piperazino]butyl]phenoxazine	62
14	10(4'-N-Pyrrolidinobutyl)phenoxazine	102
15	10-(Chloroacetyl)phenoxazine	75
16	10-[-N-Diethylamino)acetyl]phenoxazine	75
17	10-(N-Morpholinoacetyl) phenoxazine	83
18	10-(N-Piperidinoacetyl)phenoxazine	61
19	10-[[(β-Hydroxyethyl)piperazino]acetyl]phenoxazine	111
20	10-(N-Pyrrolidinoacetyl)phenoxazine	97
	Verapamil (standard)	62

N^{10}-Substituted phenoxazine or verapamil at 100 μm were incubated with KB-V1 membrane in the presence of 100 nm[^{3}H] azidopine for 20 min.

variety of anticancer drugs included in the MDR family, as well as increase intracellular drug concentrations in resistant cells. The mechanism responsible for this reversal of resistance is believed to be a competition between the modulator and cytotoxic drug for binding to the ATP-dependent efflux pump, P-gp. This direct interaction can be explored using radioactive photoactivable analogues of cytotoxic agents or modulators, which are able to selectively bind and label P-gp[17-19]. Several modulators are able to compete with this binding and to inhibit the photoaffinity labeling of P-gp by azidopine, suggesting that their interaction with P-gp is the biochemical basis for their pharmacological effects on MDR cells. It has been shown that MDR modulators with potent inhibitory effect on photoaffinity labeling of P-gp were generally the most potent MDR reversers[20]. However, restoration of drug accumulation, which represents the true target of P-gp modulation, may not be enough to reverse resistance, since other mechanisms of resistance are often encountered in MDR cells, which are not related to drug accumulation[21,22].

In a previous publication, Thimmaiah et al.[23] have reported that phenoxazine potentiated the accumulation

of vinblastine (VLB) and vincristine (VCR) in MDR KBCh^R-8-5 and GC$_3$/cl cells to a greater extent than the prototype modulator, verapamil. However, it was less effective in sensitizing the same cell lines, probably due to its instability in culture medium. In a subsequent study, twenty one N^{10} -substituted phenoxazines[24,25] were synthesized and examined for their ability to enhance the uptake of VLB and VCR in KBCh^R-8-5 and GC$_3$/cl cells. These results revealed that substitution on the phenoxazine ring at position N^{10} was associated with an increased ability to sensitize cells to VLB. The exact mechanism by which P-gp functions is still not fully understood. In order to investigate whether a relationship exists between the inhibition of azidopine binding to P-gp by MDR modulators and their efficiency in circumventing drug resistance, the present study was initiated in which the competition between [^{3}H] azidopine binding to P-gp and twenty N^{10}-substituted phenoxazines was examined.

EXPERIMENTAL

Chemistry

The synthesis and chemical characterization of twentyone N^{10}-substituted phenoxazines and their anti-MDR activity against KBCh^R-8-5 and GC$_3$/cl cells have been described by Thimmaiah et al.[24] earlier. The compounds **(1-20)** in the purified form (Table-1) are used in the present work.

Materials

All the chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. Vinblastine sulphate, verapamil hydrochloride and dimethylsulfoxide (tissue-culture grade) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). [G-^{3}H] azidopine (sp.act. 42 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL, USA). DMEM powder (with L-glutamine and without sodium pyruvate and sodium carbonate), fetal bovine serum and trypsin were purchased from Imperial Co. UK. The plastic tissue-culture wares (disposable) were purchased from Greiner GmbH, Germany.

Cell lines and cell cultures

KB-3-1, KBCh^R-8-5 and KB-V1 cells were grown in monolayer culture in antibiotic-free Dulbecco's Modified Eagle's 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 and KB-V1 cells was maintained by culturing them respectively with 10 ng/ml of colchicine and 1μg/ml of vinblastine. KBCh^R - 8-5 and KB - V1 cells overexpressed mdr 1 and were positive for P-gp as determined by immunocytochemistry using HyB 241 and C219 monoclonal antibodies.

Competition for [^{3}H] azidopine labeling of P-glycoprotein

Competition assay for photolabeling of P-gp utilized membranes from KB-V1 cells which have higher P-glycoprotein levels than do KBCh^R-8-5 cells. Crude membranes were prepared from the MDR variant, KB-V1, essentially as described previously[25]. Briefly, >10^9 KB-V1 cells were homogenized in ice-cold medium containing 20 mM Tris-base, pH 7.2, 250 mM sucrose and 0.5 mM dithiothreitol (DTT) and then centrifuged at 1000 x g to remove nuclei and unbroken cells. The supernatant was further centrifuged at 10,000 x g to separate mitochondria; then, the membrane fraction was obtained by centrifugation at 270,000 x g. This pellet was suspended in a medium containing 25 mM Tris-base pH 7.2, and 100nM mannitol and the suspension was stored at -80° until use. For photoaffinity labeling, membrane protein (200 μg) in buffer containing 250 mM sucrose and 10 mM Tris. HCl, pH 7.4 at 25°, was mixed with 100 nM [^{3}H] azidopine (42 Ci/mmol) in the absence (0.1%DMSO) or presence of 25, 50 and 100μM modulators **(1-20)** in a total volume of 150 μl. After incubating for 20 minutes in the dark, the mixture was then irradiated with a germicidal UV-light (GE Germicidal Lights, G30 T8, 30 watts), commonly used in laminar flow hoods, for 20 minutes at a distance of 10 cm.

SDS-PAGE and autoradiography

Finally, the samples were centrifuged at 270000 x g for 30 minutes and the pellets resuspended. The photolabeled proteins were separated by one-dimensional 5-15% SDS-PAGE under reducing conditions using the discontinuous buffer system of Laemmli[26]. After staining with coomassie blue and destaining, the gels were soaked in Amplify (Amersham Corp.) for 30 minutes and dried under vacuum at 75°. The dried gels were exposed to X-OMAT AR film for two to three days at-70° and developed. Radioactively labeled bands were scanned in their

centers with a densitometer (Model E-C 910, EC Apparatus Corp. St. Petersburg, FL) and quantitated by integration with a chromatography recorder (Chromatopac C-R6A, Shimadzu Corp., Kyoto, Japan).

Immunocytochemistry

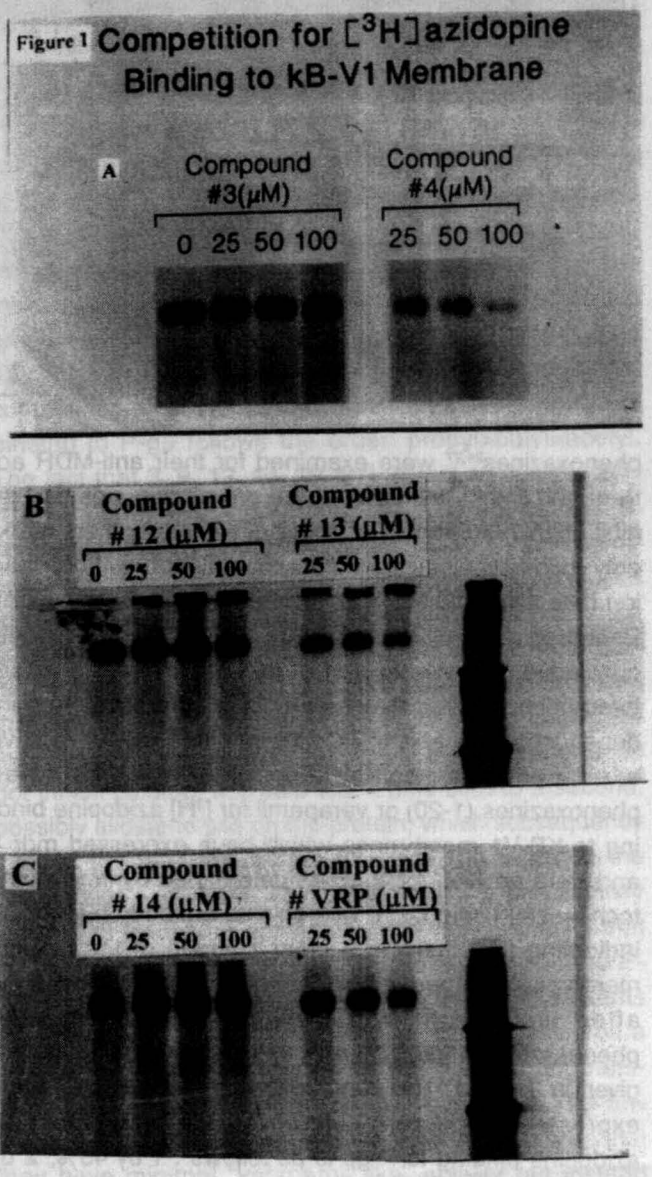
KB-V1 cells were grown on plastic chamber well slides (Four-well) at an initial cell density of 30,000/well. When the cells were in midlogarithmic growth phase, slides were washed twice with PBS and air dried overnight. Slides were then fixed in cold acetone for 10 minutes. M_o Ab C219 was used for the detection of P-glycoprotein according to Horton et al.[27]

RESULTS AND DISCUSSION

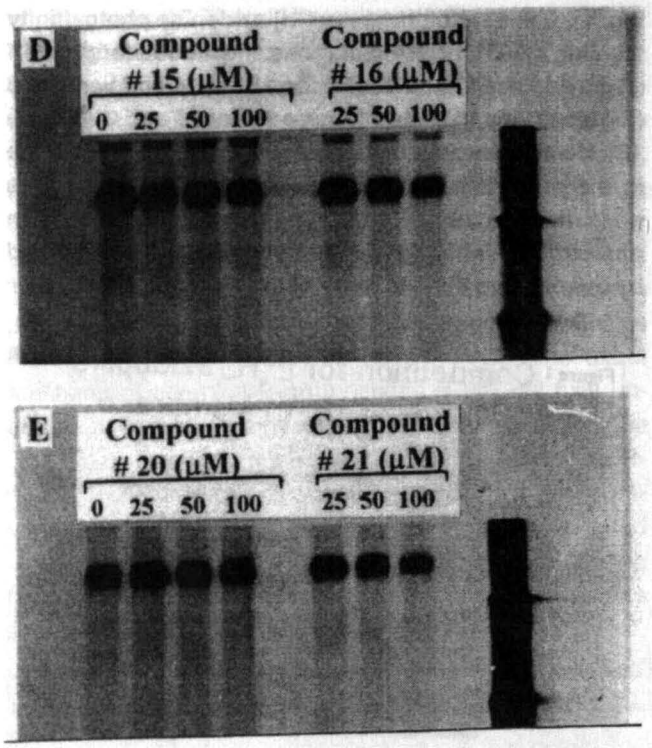
P-glycoprotein (P-gp) has been suggested to play a central role in the development of MDR by binding to drugs and exporting them by an energy-dependent process. Further, all anticancer drugs belonging to the natural product group have been proposed to be similarly transported, atleast qualitatively by this P-gp transporter. One of the most compelling pieces of experimental evidence that directly connects reduced cellular drug accumulation and active transport (pumping) of a variety of agents is found in the ability of several photoactivable compounds to covalently bind P-gp. Interestingly, these labels seem to be selective for P-gp. Also, labeling of P-gp by VLB analogues can be inhibited by addition of verapamil or other modulators of MDR.

The two most commonly used photolabeling agents which seem to predominantly label P-gp are N-(p-azido-[3-^{125}I] salicyl)-N-β-aminoethyl-vindesine and a dihydropyridine derivative, azidopine. The ability of parent phenoxazine[23] to increase the cellular accumulation of VCR and VLB in KBCh^R-8-5 and GC_3/cl cells suggests that the process was mediated through a target overexpressed in MDR cells. Cornwell[28], originally showed that P-gp of MDR KB cells is specifically labeled with VLB analogues and that verapamil blocked the specific labeling. A dihydropyridine analogue, azidopine and azidoverapamil have been found to specifically interact with P-gp by photoaffinity labelling assay. Thimmaiah et al.[23] have investigated whether parent phenoxazine interacts with P-gp by photolabeling this protein with [^{3}H] azidopine or [^{3}H] azidoverapamil in the presence of 200 to 800-fold excess of phenoxazine. The results showed

Fig. 1 : Competition between [^{3}H] azidopine photoaffinity labeling of KBV1 cell membranes and modulators 3 and 4 (Figure 1A) or 12 and 13 (Figure 1B) or 14 and verapamil (Figure 1C) or 15 and 16 (Figure 1D) or 20 and 21 (Figure 1E). Membranes were incubated with 100 nm [^{3}H] azidopine in the presence of 0, 25, 50 and 100 μM modulator as described in experimental methods. Reduction in photoaffinity labeling of P-glycoprotein was determined by quantitative densitometry of the autoradiogram.

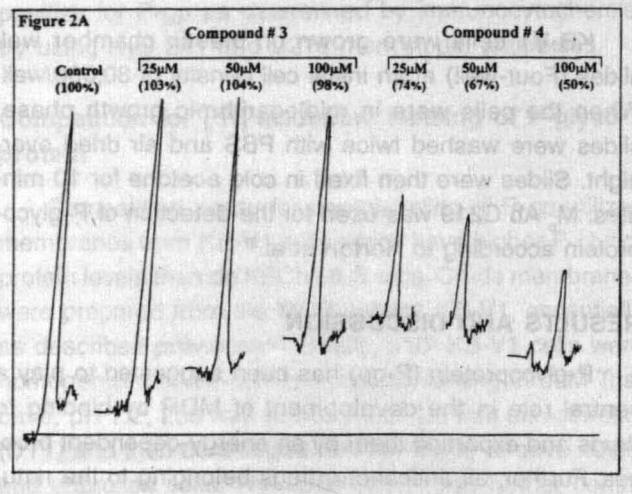


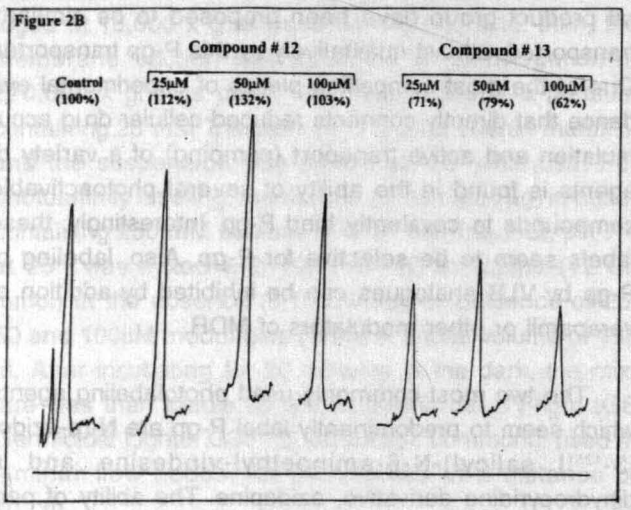
greater inhibiting efficacy of phenoxazine with azidopine than with azidoverapamil, suggesting that phenoxazine directly or indirectly affects specific moieties in MDR cells. Subsequently, twentyone N^{10}-substituted

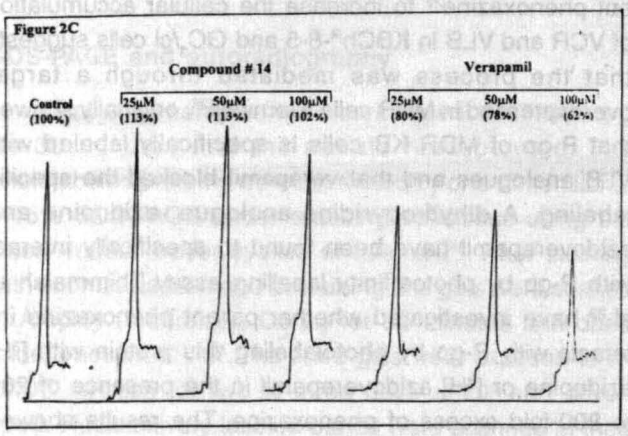


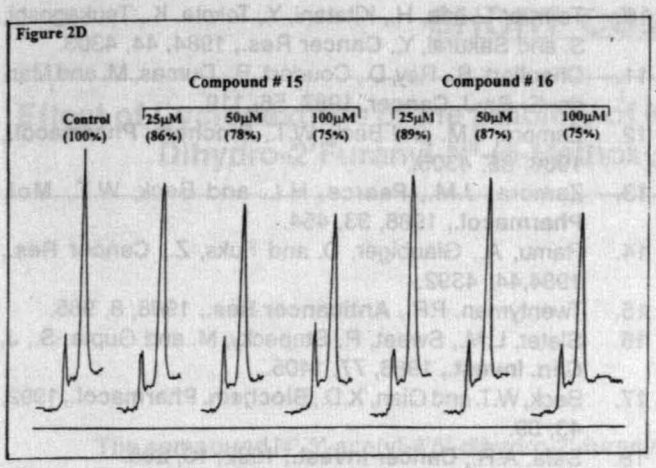
phenoxazines[24,27] were examined for their anti-MDR activity and within the series there are compounds that inhibit efflux (verapamil-like activity), whereas others markedly increase vinca alkaloid accumulation without having detectable inhibitory activity of the efflux component. Based on the uptake and efflux data, it was tentatively concluded that atleast part of the activity of N^{10}-substituted phenoxazines may be mediated through a P-gp independent mechanism. To confirm this finding, we have examined the competition by N^{10}-substituted phenoxazines (1-20) or verapamil for [^{3}H] azidopine binding to KB-V1 membranes which have expressed mdr 1 and were positive for P-gp as determined by immunocytochemistry using C219 monoclonal antibody (Figure 3), indicating that there is a clear staining of the plasma membrane. The percent binding of [^{3}H] azidopine to P-gp after incubation with individual N^{10}-substituted phenoxazines at 0,25,50 and 100 μM concentrations are given in Table 1. The competition by each phenoxazine expressed as percent control (no modulator) for [^{3}H] azidopine binding to P-gp is as follows : **1** by 45%, **2** by 30%, **3** by 2%, **4** by 50%, **5** by 29%, **6** by 64%, **9** by 4%, **10** by 24%, **11** by 45%, **12** (no competition). **13** by 38%, **14** (no competition), **15** by 25%, **16** by 25%, **17** by 17%, **18** by 39%, **19** (no competition), **20** by 3% and verapamil (standard modulator) by 38%. Data for competition

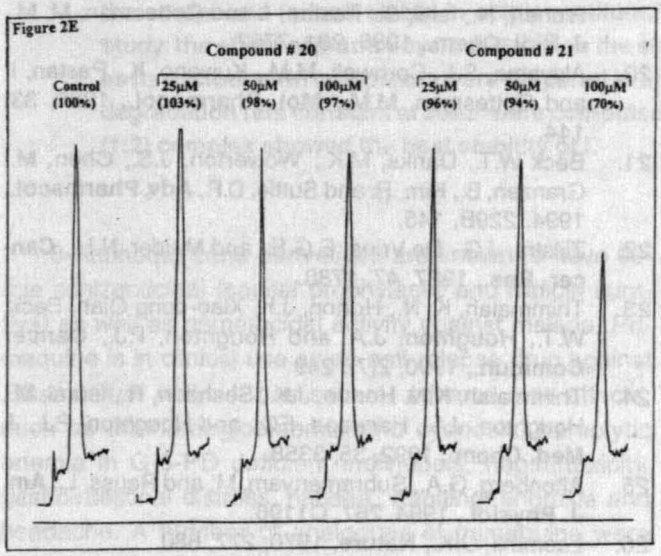
Fig. 2 : Densitogram for the modulators 3 and 4 has been shown in Figure 2A, densitogram for modulators 12 and 13 is in Figure 2B, modulator 14 and verapamil have been shown in Figure 2C. Figure 2D is for modulators 15 and 16 and modulators 20 and 21 are represented in Figure 2E



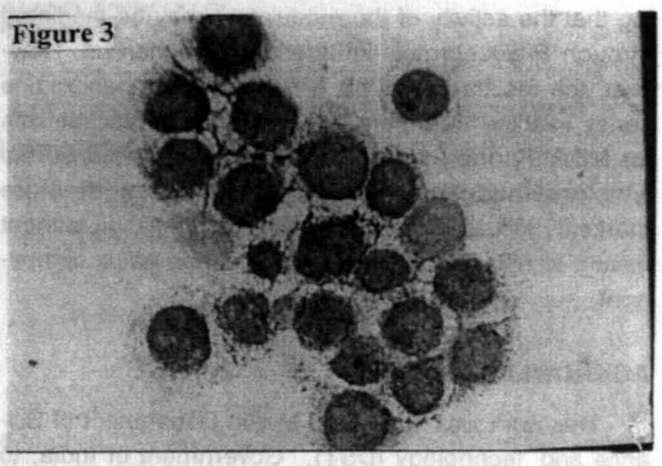








between [^{3}H] azidopine photoaffinity labeling of KB-V1 cell membranes and phenoxazines **3** and **4** (Figure 1A) or **12** and **13** (Figure 1B) or **14** and verapamil (Figure 1C) or **15** and **16** (Figure 1D) or **20** and **21** (Figure 1E) are shown. The competition of modulators for [^{3}H] azidopine binding to P-glycoprotein-rich membrane fractions isolated from MDR KB-V1 cells was assessed by scanning the radioactively labelled bands on X-OMAT AR developed film in their centres with a densitometer. The respective densitogram for the modulators **3** and **4** (Figure 2A), **12** and**13** (Figure 2B), **14** and verapamil (Figure 2C), **15** and **16** (Figure 2D) and **20** and **21** (Figure 2E) are given. Examination of the competition data shows that except modulators **3, 9, 12, 14, 19** and **20** the remaining modulators compete for azidopine binding to P-gp to varying degrees. Comparison of the data at 100



μM concentration revealed that the ability of modulators **1, 4, 6** and **11** in inhibiting the binding of [^{3}H] azidopine to P-gp is greater than that of the standard modulator, verapamil. From among the compounds tested, only two modulators 4 and **6** exhibited the maximum competition and the remaining quoted to be less than 45% excepting **12, 14** and **19** (no competition at all). Within the series of compounds examined, the competition for [^{3}H] azidopine binding to P-gp follows the order: propyl>butyl>acetyl. The fact that **3, 9, 12, 14, 19** and **20** act atleast partially independently of P-gp, is supported by their failure to compete with [^{3}H] azidopine for binding to P-gp in membrane vesicles. The explanation that is typically employed to understand the results discussed above is that there could be several drug binding sites on P-gp. If a modulator inhibits lebeling by the probe of interest then it is said that this modulator probably functions by competing for the drug binding site on the protein. If the modulator does not inhibit binding, it is said that it may bind to a second, possibly allosteric site on the protein, which subsequently inhibits the pumping action of P-gp, The fact that the compounds **1, 4, 6** and **11** have reduced the photoaffinity labeling of azidopine appreciably, the results predict that the modulators compete with azidopine for binding to P-gp, suggesting that the activity of the modulators seems to be mediated through P-gp. These results described a novel class of MDR reversing agents that are not transported by P-gp and accumulate in MDR cells. The low affinity of **3, 9, 12, 14, 19** and **20** for P-gp suggests that they have minimal "verapamil like" activity but instead exerted effect upon vinblastine influx. It could be argued that within the series, atleast six compounds (**3, 9, 12, 14, 19** and **20**) which increase vinca alkaloid accumulation but do not compete for azidopine labeling suggest-

ing that the activity of the modulators may be mediated through P-glycoprotein-independent mechanism. However, the results within the N^{10}-substituted phenoxazine series indicate that there may be multiple mechanisms for MDR. Further, these findings are in commensurate with previous observations where the modulators markedly increase vinca alkaloid accumulation without having significant inhibitory activity of the efflux component.

ACKNOWLEDGEMENTS

This work was supported by the Department of Science and Technology (DST), Government of India, to KNT. The authors wish to thank Dr. Peter, J. Houghton, St. Jude Children's Research Hospital, Memphis, Tennessee, USA, for providing some of the facilities.

REFERENCES

1. Nielsen, D. and Skovsgaard, T., **Biochim. Biophys. Acta,** 1992, 1139, 169.
2. Endicott, J.A. and Ling, V., **Ann. Rev. Biochem.,** 1989, 58, 137.
3. Gottesman, M.M. and Pastan, I., **Ann. Rev. Biochem.,** 1993, 62, 385.
4. Keizer, H.G., Schuurhuis, G. J., Broxterman, H.J., Lankelma, J., Schoonen, G.E.J., Van Rijn, J., Pinedo, H.M. and Joenje, H., **Cancer Res.,** 1989, 49, 2988.
5. Huet, S., Schott, B. and Robert, J., **Br. J. Cancer,** 1992, 65, 538.
6. Tsuruo, T., Iida, H., Tsukagoshi, S. and Sukarai, Y., **Cancer Res.,** 1981, 41, 1967.
7. Ganapathi, R. and Grabowski, D., **Cancer Res.,** 1983, 43, 3696.
8. Inaba, M. and Nagashima, K., **J. Cancer Res.,** (Gann), 1986, 77, 197.
9. Yang, C.H., Depinho, S.G., Green Berger, L.M., Arceci, R.J. and Horowitz, S.B., **J. Biol. Chem.,** 1989, 264, 782.
10. Tsuruo, T., Iida, H., Kitatani, Y., Tokota, K., Tsukagoshi, S. and Sakurai, Y., **Cancer Res.,** 1984, 44, 4303.
11. Chauffert, B., Rey, D., Coudert, B., Dumas, M. and Martin, F., **Br. J. Cancer,** 1987, 56, 119.
12. Zamora, J.M. and Beck, W.T., **Biochem. Pharmacol.,** 1986, 35, 4305.
13. Zamora, J.M., Pearce, H.L. and Beck, W.T., **Mol. Pharmacol.,** 1988, 33, 454.
14. Ramu, A., Glaubiger, D. and Fuks, Z., **Cancer Res.,** 1984,44, 4392.
15. Twentyman, P.R., **Anticancer Res.,** 1988, 8, 985.
16. Slater, L.M., Sweet, P., Stupecky, M. and Gupta, S., **J. Clin. Invest.,** 1986, 77, 1405.
17. Beck, W.T. and Qian, X.D., **Biochem. Pharmacol.,** 1992, 43, 89.
18. Safa, A.R., **Cancer Invest.,** 1992, 10, 295.
19. Shen, D.W., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I. and Gottesman, M. M., **J. Biol. Chem.,** 1986, 261, 7762.
20. Akiyama, S.I., Cornwell, M.M., Kuwano, K., Pastan, I. and Gottesman, M.M., **Mol. Pharmacol.,** 1988, 33, 144.
21. Beck W.T., Danks, M.K., Wolverton, J.S., Chen, M., Granzen, B., Kim, R. and Suttle, D.P., **Adv. Pharmacol.,** 1994, 229B, 145.
22. Zijlstra, J.G., De Vries, E.G.E. and Mulder, N.H., **Cancer Res.,** 1987, 47, 1780.
23. Thimmaiah, K. N., Horton, J.K, Xiao-dong Qian, Beck, W.T., Houghton, J.A. and Houghton, P.J., **Cancer Commun.,** 1990, 2(7), 249.
24. Thimmaiah, K.N. Horton, J.K., Seshadri, R., Israel, M., Houghton, J.A., Harwood, F.C., and Houghton, P.J., **J. Med. Chem.,** 1992, 35, 3358.
25. Altenberg, G.A., Subramanyam, M. and Reuss, L., **Am. J. Physiol.,** 1994, 267, C1196.
26. Laemmli, U.K., **Nature,** 1970, 277, 680.
27. Horton, J.K., Thimmaiah, K.N., Harwood, F.C., Kuttesch, J.D. and Houghton, P.J., **Mol. Pharmacol.,** 1993, 44, 552.
28. Cornwell, M.M., Safa A.R., Felsted, R.L., Gottesman, M.M. and Pastan, I., **Proc. Natl. Acad. Sci.,** USA, 1986, 83, 3847.