Pergamon

Biochemical Pharmacology, Vol. 49, No. 12, pp. 1851–1861, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00517-6

ALLOSTERIC REGULATION OF [^{3}H]VINBLASTINE BINDING TO *P*-GLYCOPROTEIN OF MCF-7 ADR CELLS BY DEXNIGULDIPINE

DAVID R. FERRY,* P. JOY MALKHANDI, M. A. RUSSELL and DAVID J. KERR

The University of Birmingham CRC Institute for Cancer Studies, Clinical Research Block, Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH, U.K.

(*Received* 22 *August* 1994; *accepted* 8 *November* 1994)

Abstract—Plasma membranes were prepared from the P-glycoprotein expressing human breast cancer cell line MCF-7 ADR. [^{3}H]Vinblastine bound to these membranes saturably with a B_{max} of 24 pmol/mg of protein and K_D of 23 nM. In contrast, membranes from the parent cells MCF-7 WT, which do not express P-glycoprotein, did not bind [^{3}H]vinblastine with high affinity. Cytotoxics known to be transported by P-glycoprotein inhibited the binding of [^{3}H]vinblastine, as did multidrug reversing agents including the 1,4-dihydropyridine, dexniguldipine-HCl (K_i, 15 nM). In dissociation kinetic experiments, dexniguldipine-HCl accelerated the dissociation of [^{3}H]vinblastine from P-glycoprotein, indicating a negative heterotropic allosteric mechanism of action through a drug binding site distinct from that of vinblastine. Other 1,4-dihydropyridines tested also accelerated [^{3}H]vinblastine dissociation from P-glycoprotein, however, multidrug reversing drugs of different chemical classes, including quinidine, verapamil and cyclosporin A did not. These results suggest that P-glycoprotein of MCF-7 ADR cell membranes possesses at least two drug acceptor sites which are allosterically coupled: receptor site-1 which binds vinca alkaloids, and receptor site-2 which binds 1,4-dihydropyridines such as dexniguldipine-HCl, which had the highest affinity of the tested derivatives.

Key words: dexniguldipine-HCl; P-glycoprotein; allosteric interaction; multidrug resistance; MCF-7 cells; binding assay

P-gp† is a member of the ABC super family of transporters. These transporters hydrolyse ATP to ADP + P_i, and use the energy to move substrates across the cell surface against concentration gradients. P-gp transports a wide range of cytotoxic drugs, including vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes, and peptides such as actinomycin D [1, 2]. Cells that express P-gp accumulate only small amounts of substrate cytotoxic drugs relative to control cells, and are correspondingly relatively resistant to these cytotoxics [3]. A key feature of the phenotype of P-gp expressing cells is the reversal of cytotoxic accumulation deficit by verapamil, quinidine, cyclosporin A and many seemingly unrelated non-cytotoxic drugs [4]. The reversal of accumulation deficit is also associated with resensitization to cytotoxics, and if all these criteria are met, then the P-gp expressing cells are referred to as being MDR. The reversal of P-gp mediated MDR has been exploited clinically by adding verapamil [5] or cyclosporin A [6] to chemotherapy for resistant myeloma or high-grade lymphoma.

The gene for P-gp has been cloned and is termed *mdr* 1 [7, 8]. Hydropathy analysis predicts 12 TMS arranged in bipartite symmetry, each half of the molecule having a consensus ATP binding site [9, 10]. The combination of photoaffinity labelling using the 1,4-dihydropyridine [^{3}H]azidopine with cyanogen bromide cleavage, has identified large peptide fragments that may be involved in the binding of certain drugs to P-gp [11, 12]. However, despite much structural data, the central enigma of how P-gp promiscuously transports such a broad range of substrates remains unexplained. QSARs have shown that hydrophobicity and partial volume are important variables predicting recognition by P-gp, [13], and energy transfer photoaffinity labelling, with ^{125}INA, using doxorubicin as an energy transfer agent, have been interpreted as suggesting that P-gp extracts its substrates directly out of the cell surface membrane [14]. The most widely accepted model to account for P-gp substrate promiscuity, the so-called 'hydrophobic vacuum cleaner' hypothesis, proposes that hydrophobic drugs compete at a single common P-gp binding site from which they are extracted directly. However, another mechanism that may explain P-gp substrate promiscuity is that of multiple, allosterically interacting drug binding sites.

In this manuscript the binding of [^{3}H]vinblastine to membranes from MCF-7 ADR human breast

* Corresponding author. Tel. 0121 414-3511; FAX 0121 414-3700.

† Abbreviations: P-gp, P-glycoprotein; ABC, ATP-binding cassette; MDR, multidrug resistant; TMS, transmembrane segments; QSARs, quantitative structure activity relationships; ^{125}INA, iodonaphthaleneazide; dexniguldipine-HCl, 3-methyl-5-[3,4,4-diphenyl-1-piperidinyl-propyl]-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate hydrochloride; PMSF, phenylmethylsulphonyl fluoride; 1,4-DHP, 1,4-dihydropyridine; SDS, sodium dodecyl sulphate; MDRRAs, multidrug resistance reversal agents.

cancer cells, which have a classical MDR phenotype and hyperexpress P-gp [15], is described for the first time. The kinetics of [^{3}H]vinblastine binding to P-gp are explored in detail, and the possibility of allosterically coupled drug acceptor sites is investigated with the aid of the recently described very potent 1,4-dihydropyridine MDR reversing agent dexniguldipine [16, 17].

MATERIALS AND METHODS

Materials. [^{3}H]Vinblastine (11 or 21 Ci/mmol) was from Amersham (U.K.), was stored as recommended at −20°, and was at least 98% pure. MCF-7 WT cells were obtained from ECAC and MCF-7 ADR cells were a gift from the Beatson Institute (Glasgow, U.K.). All cell culture reagents were obtained from Gibco Europe Ltd, 80 cm^2 culture flasks were from Nunc. Doxorubicin was from Farmitalia, nicardipine and nifedipine were from Sigma, azidopine was from NEN, and dexniguldipine-HCl from Byk Gulden, Germany. Other drugs and reagents were of the highest available purity from commercial sources.

Cell culture. MCF-7 WT and the drug resistant line MCF-7 ADR cells were maintained as previously described [15] in an atmosphere of 7% carbon dioxide, 93% air at 37°. Cells were grown to confluence in 80 cm^2 culture flasks and harvested using a rubber policeman.

Surface membranes. Ten flasks of cells (equivalent to 10^9 cells) were used in each membrane preparation, cells were centrifuged and the culture medium aspirated. Cells were suspended in 40 mL of ice-cold 50 mM Tris-HCl (pH 7.4), 0.1 mM PMSF, 0.1 mM EDTA (buffer A). They were disrupted with a polytron, 3 × 30 sec, at setting 5–6. The homogenate was centrifuged at 3500 *g* for 10 min. The supernatant was then centrifuged at 40,000 *g* for 20 min and resultant pellet resuspended in 10 mL of 50 mM Tris-HCl (pH 7.4) with 0.1 mM PMSF (buffer B). The final membrane pellet is enriched 3.1-fold in saturable [^{3}H]vinblastine binding relative to whole homogenate. Membranes can be stored at −80° for at least 1 year with no loss of [^{3}H]vinblastine binding activity. Protein was assayed with a Biorad kit using bovine serum albumin as standard and with appropriate detergent controls.

Binding assay. [^{3}H]Vinblastine was incubated with membranes and various drugs and ions in buffer B at 23°, unless otherwise stated, in a volume of 0.25 mL or 90 min before bound and free drug were separated by rapid filtration through Whatman GF/C filters (pre-wetted with buffer B and 0.1% BSA) which were then washed twice with 5 mL of ice-cold 20 mM Tris-HCl, 20 mM $MgCl_2$. Assays were performed in duplicates, unless otherwise indicated. Filters were dried and retained radioactivity quantitated by liquid scintillation counting. Filter blank absorption under these washing conditions accounted for <0.5% of total filtered radioactivity. In experiments using the 1,4-DHPs (nicardipine, nifedipine, dexniguldipine-HCl or nimodipine), assays were performed under sodium lighting. Assays using azidopine were performed in near darkness because this drug is an arylazide. Drug dilutions were generally done in buffer B. However, for dexniguldipine-HCl it is known that accurate dilution requires the use of 100% DMSO and glass test tubes or the potency will be underestimated by 30–100-fold [18]. As [^{3}H]vinblastine binding is sensitive to DMSO, with an EC_{50} of 2.5% (v/v), in dexniguldipine-HCl experiments the assay volume was increased to 1.0 mL, and dexniguldipine-HCl added as 2.5 μL of DMSO solution. The presence of 0.25% DMSO in the assay still inhibited ~25% of [^{3}H]vinblastine binding to P-gp. Kinetic and drug displacement assays employed 5–10 nM [^{3}H]vinblastine and 5–15 μg of membrane protein. These conditions gave 5000–10,000 dpm total binding and 2000–5000 dpm non-specific binding defined by 3 μM unlabelled vinblastine. Additional details of experiments are given in the relevant figure legends. For association experiments, reactions were started by the addition of membranes followed by incubation for various times before separation by filtration of bound and free radioligand. For dissociation experiments, [^{3}H]-vinblastine was incubated with membranes for 90 min at 23° then for a further 30 min at 12° before addition of unlabelled drugs to block the association reaction for 1–180 min before bound and free radioligand were separated. Dissociation kinetics were also explored in pseudo-infinite dilution assays where 50 μL of an equilibrium population of [^{3}H]-vinblastine/P-gp complexes were diluted rapidly into 15 mL of buffer B, or buffer B plus a drug (e.g. nicardipine) for various times. The concentration of [^{3}H]vinblastine in the equilibrium mixtures before dilution was ~25 nM (i.e. ~50% occupancy of P-gp); after dilution to ~80 pM the occupancy of P-gp by [^{3}H]vinblastine will be reduced to ~0.3%.

[^{3}H]*Vinblastine accumulation in whole cells.* For these assays, MCF-7 WT and MCF-7 ADR cells were seeded into 12-well plates and grown to confluence as previously described [15]. When the MCF-7 ADR were ~80% confluent, the medium was replaced with doxorubicin-free complete medium, and the cells grown for a further 24 hr.

Uptake assays were performed in 12-well plates (CoStar) with a well diameter of 2.2 cm as follows: the medium was aspirated and replaced with 1 mL of HEPES buffered RPMI pH 7.4, supplemented with 5 mM $MgCl_2$, 5 mM glucose, and containing 1–2 nM [^{3}H]vinblastine and unlabelled MDR reversing agents. The assay was started by the addition of [^{3}H]vinblastine to the wells. The accumulation of [^{3}H]vinblastine into both MCF-7 WT and MCF-7 ADR had reached steady state by 20 min and remained stable for at least 2 hr (not shown). Cells were incubated at 37° for 60 min after which the medium was aspirated and replaced with 1 mL of scintillant. Some workers wash cells in ice-cold PBS and others boil them in SDS prior to the addition of scintillant. In the experiments shown in this manuscript, such procedures were found not to affect the final results and were therefore omitted. After 20 min, the scintillant was transferred to scintillation vials and the radioactivity was quantitated.

Cell-free control wells demonstrated that the adsorption of [^{3}H]vinblastine to the wells constituted less than 5% of the cell retained [^{3}H]vinblastine. Data are presented in Fig. 1 as pmol of cell associated [^{3}H]vinblastine/well.

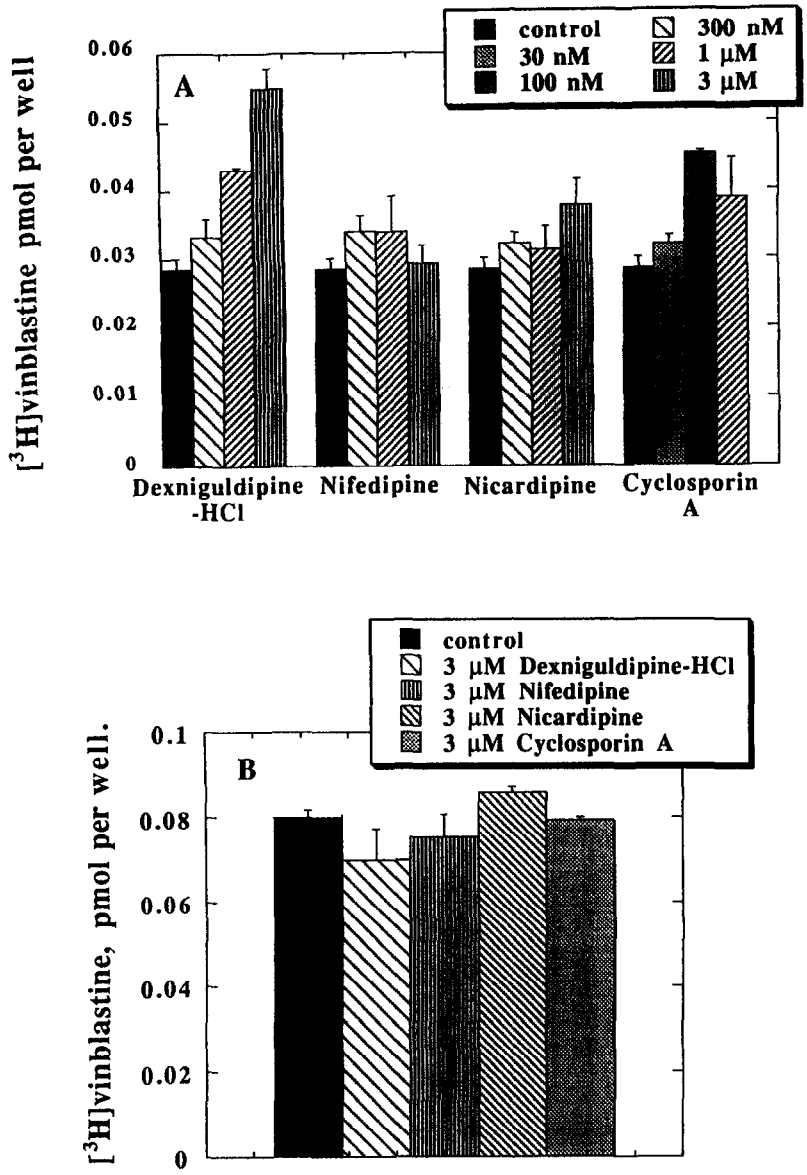


Fig. 1. Uptake of [^{3}H]vinblastine into MCF-7 ADR (A) and MCF-7 WT (B) cells. Cells were incubated at 37° for 1 hr with 1 nM [^{3}H]vinblastine and other drugs as indicated. The MCF-7 WT cells accumulated 2.62 times more [^{3}H]vinblastine than the MCF-7 ADR cells. In MCF-7 ADR cells, the accumulation deficit was reversed by dexniguldipine-HCl > cyclosporin A > nicardipine = nifedipine. In MCF-7 WT cells the accumulation of [^{3}H]vinblastine was not affected by the drugs used. Data shown are from a single representative experiment.

Data analysis. All binding data were modelled by non-linear regression using the AR module of BMDP (BMDP Statistical Software, U.S.A.) and Kaliedagraph (Ablebeck Software, U.S.A.). Binding inhibition curves for unlabelled drugs to inhibit [^{3}H]vinblastine binding were analysed by non-linear curve fitting without transformation of the data, using actual filter retained dpms. The general dose–response equation [19]:

$$Y = \left(\frac{(a-d)}{(1+[X/C]^b)}\right) + d$$

where Y is the [^{3}H]vinblastine bound to P-gp (in dpm), at a molar concentration of unlabelled drug X. The maximum of the curve is a, the minimum d (both in filter retained dpm), the slope factor b, and C is the IC_{50} for the unlabelled drug to inhibit binding. For most curves, a, b, C and d were fitted parameters. For less potent drugs, such as etoposide, d was taken as the filter retained radioactivity in the presence of 3 μM vinblastine. If $(a-d) = 100\%$, when d is filter retained dpms in the presence of 3 μM vinblastine, then the fitted value of the minimum for 1,4-DHPs (a – d_{DHP}) is in the range

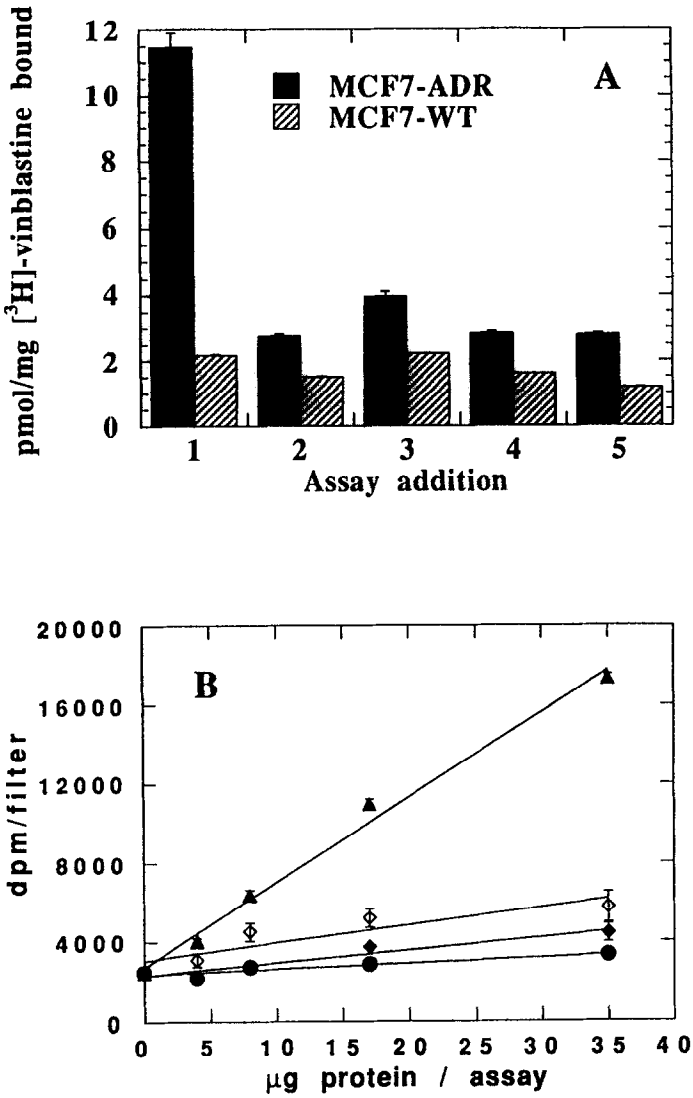


Fig. 2. Binding of [^{3}H]vinblastine to MCF-7 ADR and MCF-7 WT membranes. (A) 12 nM [^{3}H]-vinblastine was incubated with 81 μg of MCF-7 WT or 27 μg of MCF-7 ADR cell membranes for 90 min, before bound and free radioligand were separated by filtration. Each value was determined in triplicate, and error bars are shown. The assay conditions are: 1, 50 mM Tris-HCl buffer; 2, 50 mM Tris-HCl buffer plus 30 μM vinblastine; 3, 50 mM Tris-HCl buffer plus 10 μM nicardipine; 4, 50 mM Tris-HCl buffer plus 100 μM verapamil and 5, 50 mM Tris-HCl buffer plus 30 μM doxorubicin. (B) Protein-dependence of [^{3}H]vinblastine binding to P-gp of MCF-7 ADR cell membranes. MCF-7 ADR cell membranes at the amounts indicated were incubated with 11 nM [^{3}H]vinblastine for 90 min before bound and free radioligand were separated, either in buffer B alone (▲), or the presence of 30 μM vinblastine (●), 100 μM verapamil (◆) or 30 μM nicardipine (◇).

75–85%. K_i values were calculated from the Cheng and Prusoff equation [20].

For saturation analysis with radiolabelled [^{3}H]-vinblastine, data were fitted to the equation:

$$B = \left(B_{\max}\left[\frac{F^s}{F^s + K_D^s}\right]\right)$$

where B is the P-gp bound [^{3}H]vinblastine at the free (F) concentration of radioligand. The dissociation constant is K_D, $B_{\max}$ is the maximum density of sites and s is the slope factor. The concentration of free [^{3}H]vinblastine is known; the other parameters are modelled.

For the association kinetics of [^{3}H]vinblastine binding to P-gp, data were modelled to a second-order reversible reaction:

$$P + V \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} PV$$

where P is P-gp and V is [^{3}H]vinblastine, k_{-1} is the

dissociation rate constant (min^{-1}), k_{+1} the association rate constant (min^{-1} $nmol^{-1}$) and PV the P-gp/[^{3}H]-vinblastine complex using the equation:

$$\left[\frac{dPV}{dt}\right] = k_{+1}[P - PV][V - PV] + k_{-1}[PV]$$

For dissociation experiments where equilibrium had been reached and the association reaction was blocked by either an excess of unlabelled vinblastine or by 300-fold dilution, the data were modelled to a monoexponential equation. These data can be linearized by plotting the natural log of the ratio of PV complexes at time t to those at equilibrium versus time.

Parameters from modelled data of individual experiments are given with asymptotic standard deviations. Means from N experiments are given with standard errors of the mean (SEM), and statistical comparison made with Student's t-test using Statview 4 (Cherwell, Abacus Concepts). For results of individual experiments which are modelled mathematically, calculated parameters are given with asymptotic standard deviations.

RESULTS

Accumulation of [^{3}H]*vinblastine by MCF-7 ADR and MCF-7 WT cells*

The accumulation of 2 nM [^{3}H]vinblastine into MCF-7 ADR and MCF-7 WT cells reached steady state by 20 min and remained at the plateau level for 2 hr. Under the conditions employed the wild type cells accumulated approximately 2.5 ± 0.4 (mean ± SEM, N = 3)-fold more [^{3}H]vinblastine than the resistant cells. This accumulation deficit was reversed by dexniguldipine-HCl > cyclosporin A > nicardipine > nifedipine (Fig. 1). The highest concentrations of drugs employed (3 μM) did not affect the accumulation of [^{3}H]vinblastine into the MCF-7 WT cells; however, higher concentrations (>10 μM) had non-specific toxic effects, evidenced by cells floating off into medium. At 3 μM the most potent modulator tested, dexniguldipine-HCl, reversed 87 ± 12% of the accumulation deficit (N = 4). Based on the maximum accumulation seen, the IC_{50} of dexniguldipine-HCl to reverse the [^{3}H]-vinblastine accumulation deficit was 445 ± 100 nM (N = 4).

Binding of [^{3}H]*vinblastine to MCF-7ADR and MCF-7 WT cell membranes*

Binding of [^{3}H]vinblastine to resistant MCF-7ADR and sensitive MCF-7 WT cell membranes was compared in 50 mM Tris-HCl, 0.1 mM PMSF (i.e. in the absence of ATP/Mg^{2+}). MCF-7 ADR membranes bound 6 ± 1.5 (N = 3) times as much [^{3}H]vinblastine as MCF-7 WT membranes (Fig. 2A). In the presence of 30 μM unlabelled vinblastine, the amount of filter retained [^{3}H]vinblastine in MCF-7ADR membranes was close to that found in MCF-7 WT membranes. Furthermore, [^{3}H]vinblastine binding to MCF-7 ADR membranes was blocked by the cytotoxics doxorubicin (100 μM) and etoposide (100 μM), and by the MDRRAs verapamil (100 μM) and nicardipine (10 μM) (Fig. 2A). The binding of

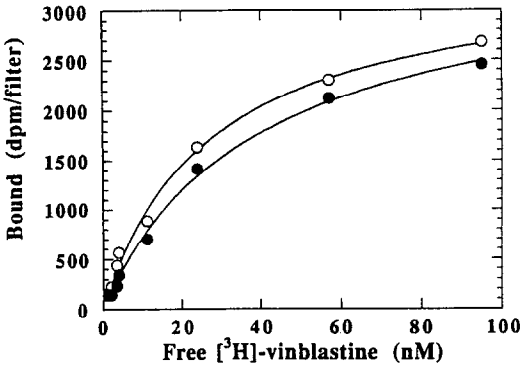


Fig. 3. Saturation isotherm for [^{3}H]vinblastine binding to P-gp of MCF-7 ADR membranes. 1.35–98 nM [^{3}H]-vinblastine was incubated with 6.5 μg of membrane protein for 90 min at 23° in buffer B (○) or in buffer B plus 1 mM ATP and 3 mM Mg^{2+} (●) before bound and free ligand were separated by filtration. Blanks measured in the presence of 3 μM vinblastine have been subtracted; they are linear with free [^{3}H]vinblastine, with a slope of 513 dpm/10 nM. The data were modelled giving the following parameter estimates (± asymptotic standard deviations): In buffer B, B_{max} = 3423 ± 147 dpm, equivalent to 28.6 ± 1.2 pmol/mg protein) and K_D = 27 ± 3 nM; in buffer B + ATP/Mg^{2+}, B_{max} = 3506 ± 157 dpm, equivalent to 29.3 ± 1.3 pmol/mg protein and K_D = 38.8 ± 4 nM.

[^{3}H]vinblastine to MCF-7 WT membranes was estimated in parallel. Although there was a small amount of 30 μM vinblastine displaceable binding, the other drugs were inactive (Fig. 2A). The binding of [^{3}H]vinblastine to MCF-7 ADR membranes was displaceable by vinblastine (30 μM), verapamil (30 μM) and nicardipine (10 μM) and was linear with protein concentration over a range of 10–150 μg/mL (Fig. 2B).

Effects of ions and nucleotides

At a single fixed concentration of [^{3}H]vinblastine (5 nM), a mixture of 1 mM ATP and 3 mM Mg^{2+} seemed to cause a minor inhibition of [^{3}H]vinblastine binding. The effects of Na^+ (1–100 mM), K^+ (1–100 mM), Ca^{2+} (0.01–3 mM), EDTA (0.01–3 mM) and glucose (5 mM) were also investigated, but these ions did not modulate the binding of [^{3}H]vinblastine (not shown). The effects of ATP/Mg^{2+} were explored in saturation experiments. In the experiment shown in Fig. 3, in the absence of ATP/Mg^{2+}, the K_D of [^{3}H]vinblastine was 27 ± 3 nM, and the B_{max} 28 ± 1.2 pmol/mg protein. In parallel, binding was measured in buffer B supplemented with 1 mM ATP, 3 mM Mg^{2+}. These conditions led to a small inhibition of [^{3}H]vinblastine binding with a K_D of 38 ± 4 nM. The saturation analysis of [^{3}H]vinblastine in buffer B was repeated in N = 5 membrane preparations, yielding a K_D of 23 ± 7 nM, and B_{max} of 24 ± 12 pmol/mg protein.

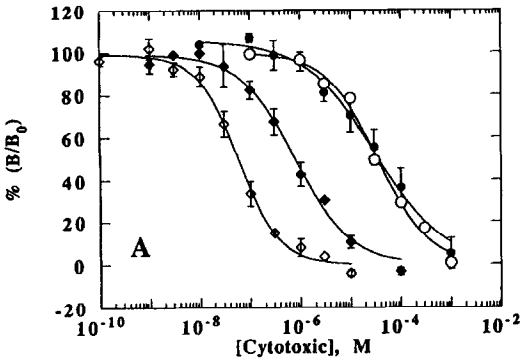
Pharmacological profile of [^{3}H]*vinblastine binding to P-gp of MCF-7 ADR membranes*

Detailed binding inhibition experiments at 10–15 nM [^{3}H]vinblastine were carried out with a wide range of cytotoxics and MDRRAs. The results are

Table 1

Drug	Slope-factor	K_i (nM)
Cytotoxics		
Vinblastine	1.20 ± 0.21	37 ± 10
Vincristine	0.95 ± 0.08	603 ± 138
Etoposide	0.75 ± 0.20	18000 ± 5000
Doxorubicin	0.87 ± 0.04	31000 ± 7300
MDDRAs		
Dexniguldipine-HCl	0.95 ± 0.23	15 ± 2.8
Azidopine	1.02 ± 0.01	71 ± 13.8
Nicardipine	1.27 ± 0.35	173 ± 49
Nifedipine	0.87 ± 0.07	7640 ± 2780
Cyclosporin A	1.10 ± 0.10	18 ± 3.6
Verapamil	1.12 ± 0.14	452 ± 50
Quinidine	0.86 ± 0.28	503 ± 183

Data are the means ± SEM from N = 3 experiments, except for nicardipine where N = 4. Data for individual dose–response curves were modelled to the general dose–response relationship by non-linear curve fitting as described in Materials and Methods. K_i values were derived from IC_{50} values by the Cheng and Prusoff equation. See Fig. 4 for selected curves.



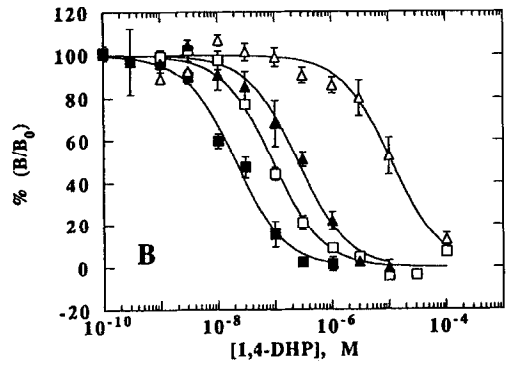
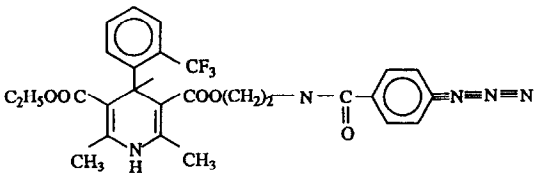
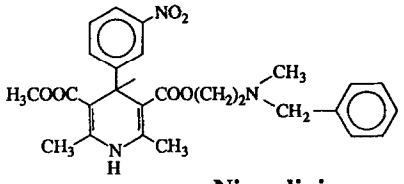


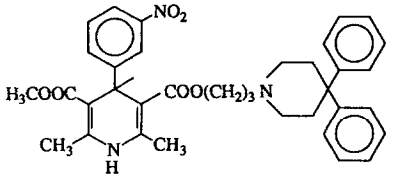
Fig. 4. Inhibition of [^{3}H]vinblastine binding to P-gp of MCF-7 ADR membranes. (A) Inhibition by cytotoxics: data are pooled from N = 3 experiments, except for vinblastine which is mean result from N = 4 experiments. (◇) Vinblastine, (◆) vincristine, (○) doxorubicin, (●) etoposide. The error bars represent SEMs. (B) 1,4-DHPs: (■) dexniguldipine-HCl, (□) azidopine, (▲) nicardipine, (△) nidefipine.



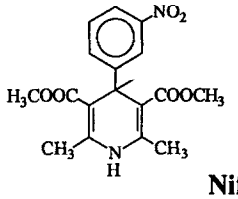
Azidopine



Nicardipine



Dexniguldipine-HCl



Nifedipine

Fig. 5. Structures of 1,4-dihydropyridines.

summarized in Table 1 and the binding inhibition curves are shown in Fig. 4 (A). The K_i value for unlabelled vinblastine was 37 ± 10 nM, which is very close to the K_D found in saturation isotherms using [^{3}H]vinblastine. The close structural analogue vincristine was considerably less potent with a K_i of 603 ± 138 nM. Other cytotoxic drugs, doxorubicin (K_i, 31 μM) and etoposide (18 μM) were far less potent.

All of the MDRRAs tested inhibited [^{3}H]-vinblastine binding to P-gp. Cyclosporin A was a potent inhibitor with a K_i of 18 ± 3.6 nM. The phenylalkylamine calcium channel blocking drug verapamil was less potent with a K_i of 452 ± 50 nM.

A number of 1,4-DHPs were tested for their ability to inhibit [^{3}H]vinblastine binding to P-gp (Fig. 4B). Nifedipine was a weak inhibitor with a K_i of 7.6 ± 2.7 μM. Nicardipine was much more potent with a K_i value of 174 ± 35 nM (N = 4). The 1,4-DHPs dexniguldipine (K_i, 15 nM) and azidopine (K_i, 71 nM) were even more potent. As can be seen in Fig. 5, dexniguldipine-HCl, azidopine and nicardipine share a common feature of having bulky 3′-substituents on the 1,4-dihydropyridine ring

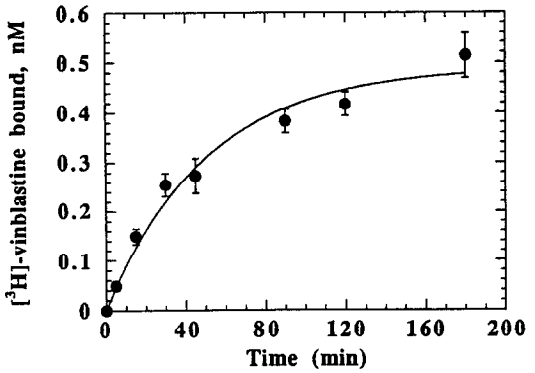


Fig. 6. Association kinetics of [^{3}H]vinblastine binding to P-gp. 11 nM [^{3}H]vinblastine was incubated with 19 μg of MCF7-ADR membrane protein, equivalent to 2.3 nM P-gp, for various times as indicated. Blank binding was defined by 3 μM vinblastine and has been subtracted, and SEM values for the triplicate determinations are shown. The data were modelled to the equation describing a reversible bimolecular reaction. The modelled parameters are (with asymptotic standard deviations): association rate constant (k_{+1}), 0.000326 ± 0.02 min^{-1} μM^{-1}; dissociation rate constant, 0.0152 ± 0.003 min^{-1}. The sum of squares was 0.05, r^2 = 0.98 and convergence was reached after 15 iterations.

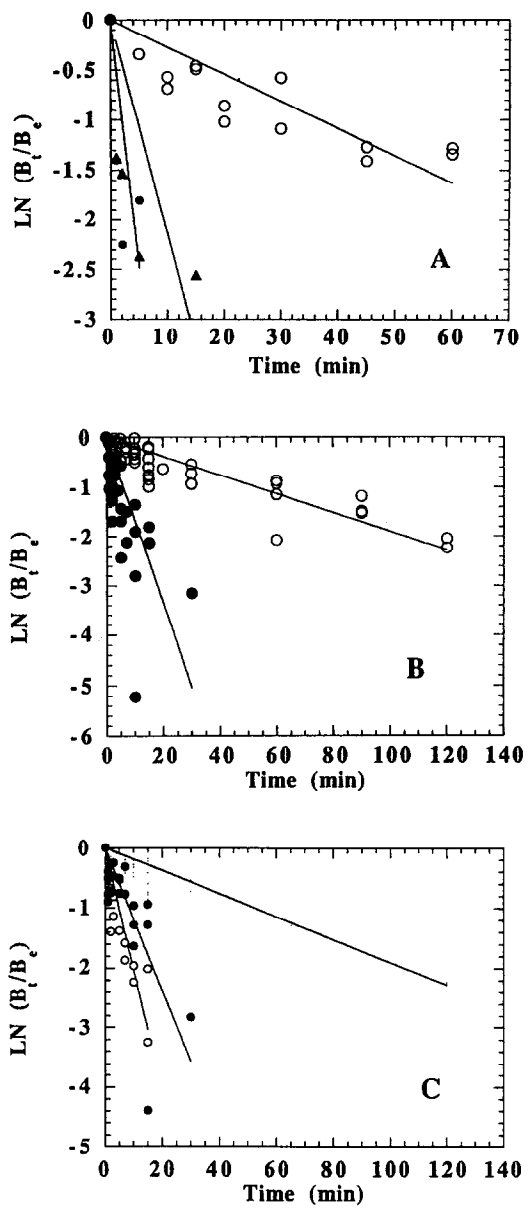


Fig. 7. Dissociation kinetics of [^{3}H]vinblastine from P-gp of MCF-7 ADR membranes. (A) By 300-fold dilution of [^{3}H]vinblastine/P-gp complexes into buffer B (○), or buffer B plus 0.3 μM nicardipine (●) or buffer B plus 3 μM nicardipine (▲). (B) By blocking the association reaction with 3 μM vinblastine, from a pool of N = 9 experiments (○) or 3 μM vinblastine plus 10 μM nicardipine, from N = 3 (●). (C) As for B, the control dissociation induced by 3 μM vinblastine is shown without data points for clarity; 3 μM vinblastine plus 30 μM nimodipine (○), 3 μM vinblastine plus 10 μM niguldipine (●). The calculated dissociation rate constants are given in Table 2.

system. This bulky substitution is clearly required for high-affinity binding in this class of MDRRAs.

Association kinetics of [^{3}H]*vinblastine to P-gp*

The association rate of 11 nM [^{3}H]vinblastine with 2.3 nM P-gp was relatively slow with 1–2 hr required for equilibrium to be reached (Fig. 6). These data were modelled to the differential form of the rate equation for a second-order reversible reaction, as described in Materials and Methods. The calculated association rate constant (k_{+1}) for N = 4 experiments was 0.45 ± 0.05 min^{-1} μM^{-1}; simultaneously k_{-1} was estimated using this method, the value calculated being 0.0192 ± 0.002 min^{-1} (N = 4). The ratio of k_{-1} to k_{+1} is the K_D which at 44 ± 8.3 nM is close to the values obtained from the displacement of [^{3}H]-vinblastine with unlabelled vinblastine, 37 nM, and from saturation analysis, 23 nM (these estimates did not significantly differ from each other).

Dissociation kinetics of [^{3}H]*vinblastine from P-gp*

If the association reaction of [^{3}H]vinblastine with P-gp is blocked by 300-fold dilution into buffer, then the rate of dissociation of [^{3}H]vinblastine from P-gp is 0.027 min^{-1}. Dilution into buffer containing nicardipine at 3 μM or at 0.3 μM accelerates the rate of dissociation by 18- and 8-fold, respectively (Fig. 7A). If 30 μM vinblastine was used to block the association reaction then ~30% of [^{3}H]vinblastine dissociated in the first 30 sec, and the remaining 70% with a half-life of 45 min (not shown). However, if 3 μM vinblastine was used to block the association reaction, then the dissociation was essentially monophasic, which makes comparative analysis under various conditions less complex. Therefore, 3 μM vinblastine was used as the standard concentration of vinblastine in dissociation experiments. Furthermore, at 23° when vinblastine/DHP mixtures were used to block the association reaction, the dissociation of [^{3}H]vinblastine from P-gp was so rapid that it could not be accurately quantified.

Table 2

Association reaction blocked by:	Dissociation rate constant (min^{-1})	Number of experiments
3 μM vinblastine	0.0211 ± 0.005	9
300-fold dilution	0.027	2
300-fold dilution + 0.3 μM nicardipine	0.214	1
300-fold dilution + 3 μM nicardipine	0.496	1
3 μM vinblastine + 10 μM dexniguldipine	0.0760 ± 0.019	3
3 μM vinblastine + 10 μM cyclosporin	0.0244	2
3 μM vinblastine + 30 μM vincristine	0.0311	2
3 μM vinblastine + 10 μM verapamil	0.0301	1
3 μM vinblastine + 30 μM quinidine	0.0186	2
3 μM vinblastine + 30 μM etoposide	0.0257	2
3 μM vinblastine + 10 μM nicardipine	0.202 ± 0.080	4
3 μM vinblastine + 30 μM nifedipine	0.078 ± 0.043	3
3 μM vinblastine + 30 μM nimodipine	0.158 ± 0.033	3

Data are mean values from the number of experiments indicated. H69/LX4 membranes were allowed to come to equilibrium with 10–15 nM [^{3}H]vinblastine for 90 min at 23° then cooled to 12° for 30 min before the association reaction was blocked by unlabelled drugs. The dilution dissociations were performed at 23° by adding 50 μL of an equilibrium population of P-gp/[^{3}H]vinblastine complexes in 15 mL of buffer for various times (see Fig. 7A). Selected examples are shown in Fig. 7.

Therefore the dissociation of [^{3}H]vinblastine from P-gp was explored in detail at 12°.

At 12°, using 3 μM vinblastine to block the association reaction, the dissociation of [^{3}H]-vinblastine was essentially monophasic with a k_{-1} of 0.0211 ± 0.005 min^{-1} (N = 9), corresponding to a half-life for [^{3}H]vinblastine/P-gp complexes of 47 min. When the association reaction was blocked with a combination of 3 μM vinblastine plus the 1,4-dihydropyridine nicardipine (10 μM), then the rate of dissociation of [^{3}H]vinblastine from P-gp accelerated by 9.6-fold (Fig. 7B). Under the same experimental conditions dexniguldipine-HCl and nimodipine also accelerated the dissociation of [^{3}H]-vinblastine from P-gp (Fig. 7C), as did nifedipine (see Table 2). In contrast, etoposide, cyclosporin A, vincristine, quinidine and verapamil did not accelerate the dissociation of [^{3}H]vinblastine from P-gp (Table 2).

DISCUSSION

Previous work has led to widespread acceptance that P-gp binds drugs competitively at a single common drug acceptor site. The data to support this conclusion came from assays in which the transport into inside-out P-gp containing vesicles of radio-labelled vinca alkaloids was measured in the presence of ATP/Mg^{2+} [21, 22]. Ideally, for the accurate determination of transport parameters, *initial rates* of transport should be measured [23]. The measurement of equilibrium accumulation of drug into a vesicle is a function of vesicle space [24], and only a small fraction of drug may be bound to P-gp. Furthermore, the nature of the competition between vinca alkaloids and other drugs was assessed by performing the saturation isotherm for [^{3}H]vinca alkaloids under control conditions and in the presence of only one concentration of unlabelled inhibitor. The data were presented as Scatchard plots [22] or Lineweaver–Burk plots [21], and because B_{max} was unchanged it was assumed that competition was occurring. The use of a single concentration of inhibitor in equilibrium assays will yield results consistent with a competitive action, however, such conclusions are likely to be premature. In such equilibrium assays, the conclusion of competitive activity can only be made when a range of concentrations has been used from which a secondary plot of slope versus [inhibitor] can be constructed [25].

The membranes we prepare from MCF-7 ADR cells are generated under low ionic strength conditions, and the [^{3}H]vinblastine binding assay performed in the absence of ATP/Mg^{2+}, indeed the presence of ATP/Mg^{2+} caused slight inhibition of binding. The binding of [^{3}H]vinblastine to P-gp is of high affinity, with a K_D of 23 nM. Control membranes from the MCF-7 WT cells, which do not express P-gp [15] did not have high affinity [^{3}H]vinblastine binding sites. The affinities of a range of P-gp cytotoxic substrates and MDRRAs for the P-gp of MCF-7 ADR membranes correlated closely with those of H69/LX4 cell membranes [26]. Previous studies have reported that nicardipine was the most potent DHP derivative to inhibit [^{3}H]vinblastine binding to P-gp, with a K_i value of 150 nM [26]. However, in this study we have compared a more extensive series of 1,4-DHPs of which dexniguldipine-HCl was the most potent having a K_i value of 15 nM. This finding correlates with the potency of dexniguldipine-HCl to reverse the MDR phenotype [16, 17]. Azidopine had a K_i value of 71 nM which is more potent that the previously reported K_D of [^{3}H]azidopine of ~1000 nM when measured in vesicle uptake assays [27].

In the [^{3}H]vinblastine accumulation assays of P-gp function in intact MCF-7 ADR cells, the

order of potency to reverse the [^{3}H]vinblastine accumulation deficit was dexniguldipine-HCl = cyclosporin A > nicardipine > nifedipine (Fig. 1). This correlates with the potency of the drugs to inhibit the binding of [^{3}H]vinblastine to membranes. However, all of the drugs tested were less potent at reversing the accumulation deficit than at displacing [^{3}H]vinblastine from P-gp in a binding assay. The estimated EC_{50} of dexniguldipine-HCl to reverse the accumulation deficit was 445 ± 100 nM (N = 4), a 30-fold lower potency than in the P-gp binding assay with [^{3}H]vinblastine where the K_i of dexniguldipine-HCl is 15 nM. The explanation for this discrepancy is not clear. In the [^{3}H]vinblastine binding assay very low concentrations of protein are used and equilibrium is reached which is important when K_i values of hydrophobic drugs are being measured [18]. In the functional assay in whole cells dexniguldipine which is highly lipophilic, may be sequestered into intracellular biophases (such as lipid) and thus the free concentration available to bind to P-gp may be much lower than the total added. The other contributing factor may be that the concentration of [^{3}H]vinblastine in the relevant cellular biophase is very high and this shifts the inhibition curves of MDRRAs to the right.

The kinetics of [^{3}H]vinblastine binding to the P-gp of MCF-7 ADR cell membranes was explored in detail. The association reaction data fitted well to a simple bimolecular model. The use of more complex models (with more variables and assumptions) was unsatisfactory as the fits did not lead to convergence. Both association rate constant (k_{+1}, $\mu M^{-1}\,min^{-1}$) and dissociation rate constant (k_{-1}, min^{-1}) were simultaneously estimated, and the ratio of these constants gave an independent estimate of K_D of 44 nM, which compares favourably with that determined by inhibition by unlabelled drug (37 nM) and equilibrium saturation with radioligand (23 nM). The dissociation rate constant estimated from modelling the association kinetics of 0.019 min^{-1} is close to that determined by 300-fold dilution of 0.027 min^{-1}. Thus the binding of [^{3}H]vinblastine to P-gp is described closely by a simple bimolecular model, with a good agreement between the K_D of [^{3}H]vinblastine determined by equilibrium and kinetic methods. According to the law of mass action when P-gp and [^{3}H]vinblastine are in equilibrium if a competitive inhibitor is introduced into the system, it can only occupy P-gp when [^{3}H]vinblastine has dissociated. Thus the dissociation rate of [^{3}H]-vinblastine from P-gp should be the same when induced by a second ligand as that found by dilution. However the 1,4-DHPs tested accelerated the dissociation rate of [^{3}H]vinblastine by 3.6 (dexniguldipine and nifedipine) to 9.6-fold (nicardipine).

The simplest explanation of these findings is that 1,4-DHPs bind to a drug acceptor site (drug acceptor site-2) which is distinct from that which binds [^{3}H]vinblastine (drug acceptor site-1). Thus when P-gp binds [^{3}H]vinblastine, a 1,4-DHP can still bind and cause a conformational change in P-gp such that the affinity for [^{3}H]vinblastine is decreased. The equations below depict this interaction between P-gp (P), vinblastine (V) and dexniguldipine (Dn)

$$P + V \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} PV \quad (1)$$

$$PV + Dn \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} PVDn \quad (2)$$

$$PVDn \underset{k_{+3}}{\overset{k_{-3}}{\rightleftarrows}} PDn + V \quad (3)$$

The formation of a ternary PVDn complex requires that P-gp is capable of simultaneously binding both vinblastine and dexniguldipine, and this requires at least two distinct binding sites. Thus 1,4-DHPs are negative heterotropic allosteric regulators of [^{3}H]-vinblastine binding to P-gp. There are many examples of transmembrane structures possessing allosterically coupled drug acceptor sites, including the nicotinic acetylcholine receptor [28], the benzodiazepine-chloride ionophore [29], the voltage-dependent sodium channel [30] and the voltage-dependent calcium channel [31]. If we accept that P-glycoprotein is a drug acceptor with allosterically coupled drug acceptor sites, then in analogy to the acetyl choline receptor [32], the question arises as to which conformation [^{3}H]vinblastine has bound to in the assay conditions employed?

In classical allosteric theory, interactions take place between subunits of macromolecules [33]. However, there are many examples of one large transmembrane structure possessing more than one drug acceptor site, e.g. the voltage-dependent L-type calcium channel [34]. Some preliminary evidence with detergent solubilized membranes and sucrose density centrifugation suggests that P-gp can form dimers [35], raising the question of allosteric interactions occurring between P-gp oligomers. However, the oligomers so far detected have not been identified by an assay of functional activity (binding or transport), but by Western blotting. It would be of great interest to know if the allosteric interactions described in particulate membranes are preserved in detergent solubilized P-gp, and if they are seen in monomeric or oligomeric P-gp. These experiments are currently underway.

Very few data are available concerning the detailed kinetics of transport of substrates by P-gp. Part of the difficulty with such transport kinetic studies is that for hydrophobic drug molecules the intracellular free drug concentration cannot easily be measured. However, using daunomycin it is possible to measure the cytosolic-free concentration [36] and demonstrate that transport of substrates is saturable. This has allowed accurate determinations of the transport K_m, which was found to be 1.5 μM, but the Hill slope for transport was 1.5–2 indicating, as the authors suggest, co-operativity. We did not find that doxorubicin accelerated vinblastine dissociation from P-glycoprotein, which suggests that perhaps vinca alkaloids and anthracyclines share a common drug acceptor site. However, this question would be better approached by finding an anthracycline of high enough affinity (1000-fold higher than doxorubicin) to allow reversible binding kinetics to be performed as we have demonstrated for [^{3}H]-vinblastine in this manuscript.

The question of where the allosterically coupled drug acceptor sites of P-gp are located at the molecular level cannot be answered by simple binding and kinetic experiments. Already, however, there are reports in the literature in which the transmembrane segments of incorporation of photoaffinity ligands have been identified. Overall they all seem to indicate that transmembrane segments 5, 6, 11 and 12 are locations to which azidopine [11] and azidoprazosin [12] bind. However, most of the photoaffinity probes are flexible arylazides, which: (i) are highly reactive when photoactivated; and (ii) due to their flexibility and because the photoreactive group is not located in a key pharmacophoric area of the ligands, may be incorporating covalently at sites distant from their binding site [12, 37]. Furthermore, because most of the photoaffinity labelling studies were performed in 'transport' buffer, the probes may be labelling not their binding site, but part of the pore through which they pass to be transported. The discovery of allosterically coupled drug acceptor sites may allow the synthesis of new photoaffinity probes in which the photoreactive groups are in pharmacologically important areas, conferring drug acceptor-site specificity

Acknowledgements—The authors are grateful to the Special Trustees of the United Hospitals of Birmingham, and the Cancer Research Campaign for funding.

REFERENCES

1. Endicott JA and Ling V, The biochemistry of multidrug resistance. *Annu Rev Biochem* **58**: 137–171, 1989.
2. Gottesman MM and Pastan P, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**: 385–427, 1993.
3. Tsuruo T, Tsukagoshi S and Sakurai Y, Overcoming vincristine resistance in P388 leukaemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* **41**: 1967–1972, 1981.
4. Bradley G, Juranka PF and Ling V, Mechanisms of multidrug resistance. *Biochem Biophys Acta* **948**: 87–128, 1988.
5. Miller TP, Grogan TM, Dalton WS, Spier CM, Scheper RJ and Salmon SE, P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high dose verapamil. *J Clin Oncol* **9**: 17–24, 1991.
6. Sonneveld P, Durie BGM, Lokhorst HM, Marie J-P, Solbu G, Zittoun R, Lowenberg B and Nooter K, Modulation of multidrug resistant myeloma by cyclosporin. *Lancet* **340**: 255–258, 1992.
7. Chen C-J, Chin JE, Ueda K, Clark DP, Gottesman MM and Roninson IB, Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug resistant cells. *Cell* **47**: 381–389, 1986.
8. Gros P, Croop J and Houseman DE, Mammalian multidrug resistance gene: Complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell* **47**: 371–380, 1986.
9. Zhang JT and Ling V, Study of membrane orientation and glycosylated extracellular loops of mouse P-glycoprotein by in vitro translation. *J Biol Chem* **266**: 18224–18232, 1991.
10. Yoshimura A, Kuwazuru Y, Sumizawa T, Ichikawa M, Ikeda S, Uda T and Akeyama S, Cytoplasmic orientation and two-domain structure of the multidrug transporter, P-glycoprotein, demonstrated with sequence-specific antibodies. *J Biol Chem* **264**: 16282–16291, 1989.
11. Bruggemann EP, Currier SJ, Gottesman MM and Pastan I, Characterisation of the azidopine and vinblastine binding site of P-glycoprotein. *J Biol Chem* **267**: 21020–21026, 1992.
12. Greenberger LM, Major photoaffinity drug labelling sites for iodaryl azidoprazosin in P-glycoprotein are within or immediately C-terminal to transmembrane domains six and twelve. *J Biol Chem* **268**: 11417–11425, 1993.
13. Zamora JM, Pearce HL and Beck WT, Physical chemical properties shared by compounds that modulate multidrug resistance in human leukaemic cells. *Mol Pharmacol* **33**: 454–462, 1988.
14. Raviv Y, Poland HB, Bruggemann EP, Pastan I and Gottesman MM, Photosensitive labelling of a functional multidrug transporter in living drug resistant tumour cells. *J Biol Chem* **265**: 3975–3980, 1990.
15. Fairchild CR, Ivy SP, Kao-Shan C-S, Whang-Peng J, Rosen N, Israel MA, Melera PW, Isolation of amplified and overexpressed DNA sequences from adriamycin-resistant human breast cancer cells. *Cancer Res* **47**: 5141–5148, 1987.
16. Reymann A, Looft G, Woermann C, Dietel M and Erttman R, Reversal of multidrug resistance in Friend leukaemia cells by dexniguldipine-HCl. *Cancer Chemother Pharmacol* **26**: 205–210, 1993.
17. Ise W, Hogg M, Sanders K-H and Geckler V, Reversal of resistance to taxol and taxotere by dexniguldipine-HCl. *Proc Am Assoc Cancer Res* **35**: 2123, 1994.
18. Boer R, Grassegger A, Schudt C and Glossmann H, (+)-Niguldipine binds with very high affinity to Ca^{2+} channels and to a sub-type of alpha l-adrenoceptors. *Eur J Pharmacol* **172**: 131–145, 1989.
19. De Lean A, Munson PJ and Rodbard D, Simultaneous analysis of families of sigmoid curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am J Physiol* **4**: E97–E104, 1978.
20. Cheng Y and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
21. Naito M and Tsuruo T, Competitive inhibition by verapamil of ATP-dependent high affinity vincristine binding to the plasma membrane of multidrug-resistant K562 cells without calcium ion involvement. *Cancer Res* **49**: 1452–1455, 1989.
22. Horio M, Gottesman MM and Pastan I, ATP-dependent transport of vinblastine in vesicles from multidrug resistant cells. *Proc Natl Acad Sci USA* **85**: 3580–3584, 1988.
23. Berteloot A and Semenza G, Advantages and limitations of vesicles for the characterisation and kinetic analysis of transport systems. *Methods Enzymol* **192**: 409–437, 1990.
24. Mircheff AK, Krippen I, Hirayama B and Wright EM, Delineation of sodium stimulated amino acid transport pathways in rabbit kidney brush border vesicles. *J Membrane Biol* **64**: 113–122, 1981.
25. Segel IH, *Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, Wiley-Interscience, New York, U.S.A., 1975.
26. Ferry DR, Russell MA and Cullen MH, P-glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is allosterically coupled to a vinca alkaloid-selective binding site. *Biochem Biophys Res Commun* **188**: 440–445, 1992.
27. Tamai I and Safa AR, Azidopine non-competitively

interacts with vinblastine and cyclosporin A binding to P-glycoprotein in multidrug resistant cells. *J Biol Chem* **266**: 16796–16800, 1991.
28. Galzi J-L, Revah F, Beiss A and Changeux JP, Functional architecture of the nicotinic acetyl choline receptor: From the electric organ to the brain. *Annu Rev Pharmacol Toxicol* **31**: 37–72, 1991.
29. Stephenson FA, Understanding the $GABA_A$ receptor: A chemically gated ion channel. *Biochem J* **247**: 21–32, 1989.
30. Catterall WA, Structure and function of voltage sensitive ion channels. *Science* **242**: 50–61, 1988.
31. Glossmann H and Striesnig J, Molecular properties of calcium channels. *Rev Physiol Biochem Pharmacol* **114**: 1–92, 1990.
32. Heidmann T, Beuchardt J, Neumann E and Changeux JP, Rapid kinetics of agonist binding and permeability response analysed in parallel on acetylcholine receptor rich membranes from *Torpedo marmorata*. *Biochemistry* **22**: 5452–5459, 1983.
33. Monod J, Changeux JP and Jacob F, Allosteric proteins and molecular control systems. *J Mol Biol* **6**: 306–329, 1963.
34. Striessnig J, Goll A, Moosburger K, Ferry DR and Glossmann H, Stereoselective photoaffinity labelling of the purified 1,4-dihydropyridine receptor of the voltage-dependent calcium channel. *Eur J Biochem* **161**: 603–609, 1986.
35. Poruchynsky MS and Ling V, Detection of oligomeric forms of P-glycoprotein in multidrug resistant (MDR) mammalian cell membranes. *Resistance against Anticancer Drugs: Molecular Mechanisms and Clinical Opportunities*. B7. 1993.
36. Spoelstra EC, Westerhoff HV, Dekker H and Lankelma J, Kinetics of daunorubicin transport by P-glycoprotein of intact cells. *Eur J Biochem* **207**: 567–579, 1992.
37. Ferry DR, Rombusch M, Goll A and Glossmann H, Photoaffinity labelling of Ca^{2+} channels with [^{3}H]-azidopine. *FEBS Lett* **169**: 112–118, 1984.