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P-GLYCOPROTEIN POSSESSES A 1,4-DIHYDROPYRIDINE-SELECTIVE DRUG ACCEPTOR SITE WHICH IS ALLOSERICALLY COUPLED TO A VINCA-ALKALOID-SELECTIVE BINDING SITE

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SUMMARY: [^{3}H]Vinblastine bound with high affinity to surface membranes prepared from H69/LX4 cells which express P-glycoprotein (P-gp) and as a consequence are multidrug resistant (MDR).The K_D was 9.8 $\pm$ 1.5 nM and density of sites 31.2 $\pm$ 8.6 pmol/mg of protein. [^{3}H]Vinblastine binding was inhibited by cytotoxics and agents known to reverse MDR. 1,4-Dihydropyridine MDR reversing agents including nicardipine and nifedipine accelerated the dissociation of [^{3}H]vinblastine from P-gp indicating a negative heterotropic allosteric effect. Cyclosporin A, vincristine and actinomycin D did not alter [^{3}H]vinblastine dissociation kinetics. It is concluded that P-gp possesses at least two allosterically coupled drug acceptor sites, receptor site-1 that is selective for vinca alkaloids and cyclosporin A, and receptor site-2 that is selective for 1,4-dihydropyridines.

The phenomenon of broad cross resistance of tumour cell lines to a variety of structurally diverse cytotoxics was first described in 1970 by Biedler and Riehm (1). Such cells demonstrate a decreased cytotoxic drug accumulation and over expression of a glycoprotein ATPase of molecular weight 170 kDa, known as P-glycoprotein (P-gp), (2). The phenotype of cells expressing P-gp is now widely referred to as multidrug resistance (MDR). P-gp is known to be located on human chromosome 7, the gene has an open reading frame of 1280 amino acids, and there are predicted to be twelve transmembrane regions and two consensus ATP-ase sites (2). Because many chemically diverse substrates and blockers of P-gp are highly hydrophobic it has been suggested that P-gp can act as a 'hydrophobic vacuum cleaner' (2), but how can P-gp recognise so many structurally diverse substrates ?

We have prepared surface membranes from the well characterised P-gp expressing human lung cancer cell line H69/LX4 (3,4) and characterised [^{3}H]vinblastine binding to these. We have used the kinetics of [^{3}H]vinblastine binding as a method of investigating if cytotoxic substrates and MDR-reversing agents bind competitively to a common single drug acceptor site of P-gp as required by the hydrophobic vacuum cleaner model.

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Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance.

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MATERIALS AND METHODS.

Materials. [^{3}H]Vinblastine (8.3-16 Ci/mmol) was from Amersham UK. H69/P and H69/LX4 cells were a gift from Dr Twentyman, Cambridge, UK. All cell culture reagents were obtained from Gibco Europe Ltd, 75 cm^2 culture flasks were from Falcon. Doxorubicin was from Farmitalia, nicardipine and nifedipine were gifts from Professor Glossmann, Innsbruck, Ro 11 2933 (N-(3,4-dimethoxyphenethyl)-N-methyl-2-(2-napthyl)-m-dithane-2-propylamine hydrochloride) was a gift from Hoffmann La Roche, Basal, Switzerland. Other drugs and reagents were of the highest available purity from commercial sources.

Cell Culture. H69/LX4 cells were grown as previously described (3) in the presence of 760 nM doxorubicin and maintained in an atmosphere of 8% carbon dioxide, 92% air at 37°C. Cells were grown in Techne culture flasks to a density of 1-2 x 10^8 cells in 400 mL of medium then harvested at 7 days. H69/P cells were grown in the absence of doxorubicin.

Surface Membranes. 1-2 x 10^8 cells were used in each membrane preparation. H69/LX4 cells were centrifuged and the culture medium decanted off. Cells were suspended in 40 ml of ice cold 50 mM Tris HCl (pH 7.4), 0.1 mM phenylmethylsulphonylfluoride (PMSF), 0.1mM EDTA (buffer A). They were disrupted with a polytron, 3 x 30 seconds, at setting 5-6. The homogenate was centrifuged at 3500 x g for 10 min. The supernatant was then centrifuged at 40 000 x 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). Membranes can be stored at -80°C for at least 1 year with no loss of [3H]vinblastine binding activity. Protein was assayed with a Biorad kit using bovine serum albumin as standard.

Binding Assay. [^{3}H]Vinblastine was incubated with membranes and various drugs and ions in buffer B at 12 or 23°C in a volume of 0.25 mL for 60 min before bound and free drug were separated by rapid filtration through Whatman GF/C filters which were then washed twice with 5 ml of ice-cold 20 mM Tris HCl, 20 mM Mg Cl_2. Assays were performed in duplicates. Filters were dried and retained radioactivity quantitated by liquid scintillation counting. In experiments using the 1,4-dihydropyridines (nicardipine nifedipine, nitrendipine or nimodipine), assays were performed under sodium lighting. Kinetic and drug displacement assays employed 5-10 nM [^{3}H]vinblastine and 5-15 μg of membrane protein. These conditions gave 2500-5000 dpm total binding and 500-1600 dpm nonspecific binding defined by 30 μM unlabelled vinblastine. Additional details of experiments are given in the relevant figure legends.

Data analysis. Dose response curves were analysed by non-linear curve fitting procedures using the general dose-response equation (5), saturation analyses were analysed by non-linear curve fitting to the logistic model. K_i values were calculated according to Cheng and Prussof (6). Statistical significance was assessed using Student's two tailed t-test.

RESULTS. In membranes from H69/LX4 cells [^{3}H]vinblastine bound to a single population of high affinity sites with a K_D of 9.8 ± 1.5 nM and B_{max} of 31.2 ± 8.6 pmol/mg of protein (n = 5 membrane preparations). As shown in figure 1A at [^{3}H]vinblastine concentrations as high as 12 times K_D there was no suggestion of the presence of a low affinity site. Membranes from P-gp expressing H69/LX4 cells bound approximately 20-fold more [^{3}H]vinblastine over the concentration range 2-17 nM, than those from H69/P cells which do not express P-gp (4), (fig 1B). [^{3}H]Vinblastine binding to H69/LX4 membranes was not ATP or Mg^{2+} dependent. Indeed 5 mM ATP inhibited [^{3}H]vinblastine binding by 30%. Sucrose at 250 mM inhibited binding by 25%, and the combination of 2mM Mg^{2+}, 5 mM ATP and 250 mM sucrose inhibited binding by 20-30%.

A number of cytotoxics displaced [^{3}H]vinblastine binding. The rank order of potency for these drugs was vinblastine > vincristine > actinomycin D > doxorubicin > etoposide > colchicine (see table I for K_i values). Both vincristine and actinomycin D displaced to the same level as 30 μM vinblastine which is used to define non-receptor binding (fig 2A). Owing to their low affinity doxorubicin and etoposide did not displace 100% of vinblastine bound to P-gp at their limits of solubility. In contrast to other potent drugs tested, the 1,4-dihydropyridines

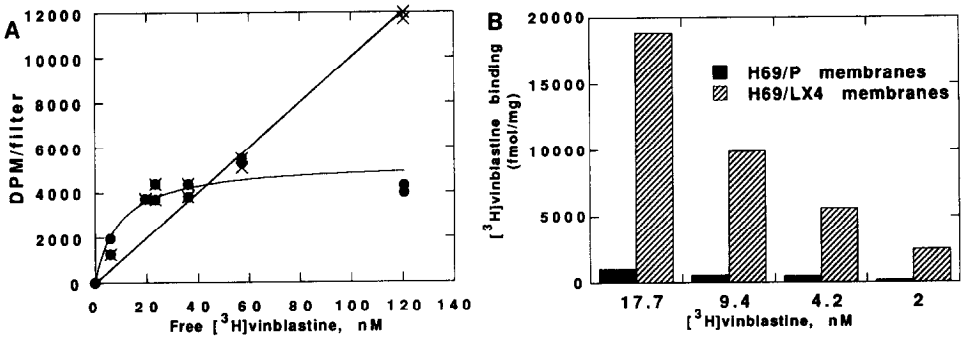

Figure 1. (A) Saturation analysis of [3H]vinblastine binding to membranes from H69 LX4 cells. This assay was performed in 0.1 ml to allow high concentrations of radioligand to be used. Specific (•) and blank binding defined by 30 μM vinblastine (x) are shown. Specific binding was analysed with non-linear curve fitting, the line drawn is the best fit with the parameters, K_D = 9.4 ± 3.3 nM, B_{max} = 5312 ± 448 dpm, r = 0.930, as 7 μg of protein was employed the B_{max} = 32 pmol/mg membrane protein. (B) Binding of [3H]vinblastine at indicated concentrations to membranes from H69/LX4 (hatched bars) and H69/P (filled bars) cells as described in methods.

displaced only 80-85% of [3H]vinblastine binding to P-gp. An example with nicardipine is shown in fig 2B, where over the range 1 to 30 μM binding inhibition plateaus at 85%, whereas in contrast verapamil (a phenylalkylamine) can inhibit 100% of [3H]vinblastine binding to P-gp (fig 2B).

Dissociation of an equilibrium population of [3H]vinblastine/P-gp complexes at 23°C induced by a mixture of 30 μM vinblastine/10 μM nicardipine was too rapid to accurately quantify (not shown). Subsequent dissociation experiments were therefore performed at 12°C. At 12°C the dissociation of [3H]vinblastine/P-gp complexes induced by 30 μM vinblastine occurred with a dissociation rate constant (k_{-1}) of 0.0357 ± 0.005 min^{-1} (n = 7). The mixture of 30 μM vinblastine and the 1,4-dihydropyridine nicardipine induced a 11-fold more rapid

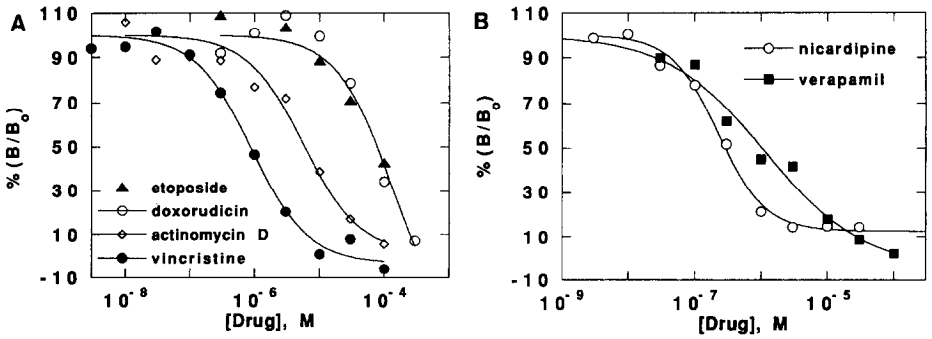

Figure 2. (A) Displacement of [3H]vinblastine binding from H69/LX4 membranes by cytotoxics. Data points are means (n = 3) experiments, except for etoposide (n = 2). (B) Displacement of [3H]vinblastine binding by verapamil (n = 3) and nicardipine (n = 5). B_0 is specific binding in the absence of added drug and B in the presence of drugs at indicated concentrations, see table 1 for K_i values and slope factors.

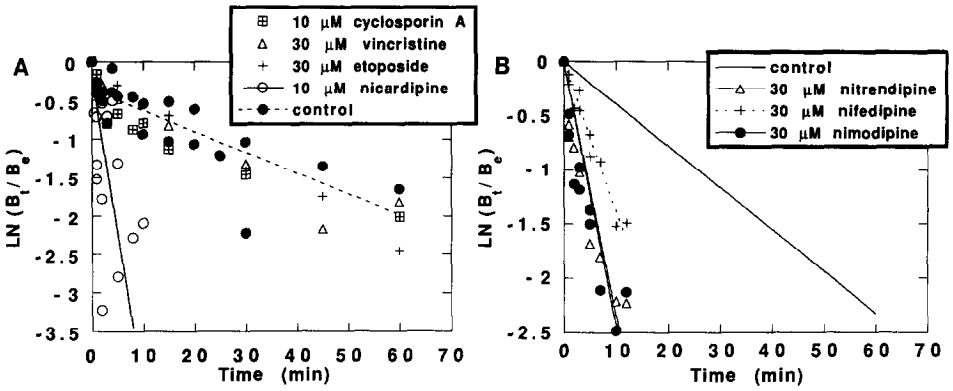

Figure 3. Dissociation kinetics of [^{3}H]vinblastine binding from P-gp. (A) The association reaction between P-gp and [^{3}H]vinblastine was blocked by 30 μM vinblastine (control), or combined with other drugs as indicated by symbols in the insert. B_e, specifically bound [^{3}H]vinblastine at equilibrium and B_t, t min after blockade of the association reaction by drugs as indicated. The data points for control with 30 μM vinblastine and control plus 10 μM nicardipine are pooled from three separate experiments, the lines were drawn by linear regression analysis. The other experiments were performed separately and are representative examples. For clarity regession lines have not been drawn for these experiments, but see table 1 for rate constants. (B) As for A, the line drawn for control with 30 μM vinblastine is the fit derived from n = 7 experiments for which $k_{-1} = 0.0357$ min^{-1}, for clarity data points have been omitted. Data points with indicated 1,4-dihydropyridines are pooled results from two experiments. Dissociation rate constants are given in table 1.

dissociation with a k_{-1} of 0.393 ± 0.11 min^{-1} (n = 3, p = 0.001). Other 1,4-dihydropyridines at 30 μM, (nifedipine, nimodipine and nitrendipine) increased the dissociation rate of [^{3}H]vinblastine/P-gp complexes 4 to 7-fold (fig 3B and table I). In combination with 30 μM

Table 1
DRUG INHIBITION KINETICS

	Ki (nM)	Slope factor	Dissociation rate (min^{-1})	[Drug] (μM)
Cytotoxics				
Vinblastine	9 ± 2	1.02 ± 0.10	0.0375 ± 0.005[a]	30
Vincristine	600 ± 180	1.13 ± 0.10	0.0330	30
Actinomycin D	3020 ± 1540	0.89 ± 0.10	0.0376	30
Doxorubicin	18200 ± 4500	1.17 ± 0.10	0.0630 ± 0.004[e]	300
Etoposide	20100	-	0.0390	300
Colchicine	>100000	-	ND	ND
MDR reversing agents				
Cyclosporin A	17 ± 2.2	1.24 ± 0.16	0.0255 ± 0.066	10
Ro 11 2933	28 ± 14	0.62 ± 0.08	0.0640	1
Verapamil	600 ± 180	0.78 ± 0.06	0.0600 ± 0.050[f]	10
Quinidine	274 ± 119	0.73 ± 0.05	0.0690	10
Quinine	1912 ± 473[c]	0.90 ± 0.07	ND	ND
1,4-dihydropyridines				
Nicardipine	153 ± 47[b]	0.95 ± 0.10	0.393 ± 0.110[d]	10
Nimodipine	1000	1.60	0.243	30
Nitrendipine	1170	1.21	0.233	30
Nifedipine	5000	1.28	0.138	30

Dissociation was induced by 30 μM vinblastine, plus other drugs added in addition at indicated concentrations. Values with SEMs are means of 3 experiments, those values without SEMs are means of 2 experiments which differed by less than 10%, other than, [a]mean ± SEM for n = 8, [b]mean ± SEM for n = 5, [c]$p < 0.05$ relative to K_i for quinine, [d]$p < 0.001$ relative to k_{-1} induced by 30 μM vinblastine, [e]$p < 0.05$ relative to k_{-1} with 30 μM vinblastine, [f]$p < 0.05$ relative to k_{-1} with 30 μM vinblastine, ND not determined.

vinblastine the anthracycline doxorubicin (at 300 μM) and the phenylalklamine verapamil (at 30 μM) increased the dissociation rate of [^{3}H]vinblastine/P-gp complexes 2-fold, whereas vincristine, etoposide, and cyclosporin A did not (see table I for rate constants and fig 3A).

In the presence of increasing concentrations of unlabelled nicardipine the apparent K_D of [^{3}H]vinblastine increased, but at 1 μM the nature of inhibition is not simply competitive, with a decrease of B_{max} of 22 ± 7% ($p < 0.05$). In contrast cyclosporin A did not cause a decrease in B_{max} in the binding of [^{3}H]vinblastine to P-gp (not shown).

DISCUSSION. In agreement with previous work we found that binding of [^{3}H]vinblastine to P-gp was not dependent on ATP, and found a density of binding sites of 31 pmol/mg protein, which is close to that found in membranes from KB-C4 cells of 40 pmol/mg protein (7). Other authors have prepared inside-out vesicles from P-gp expressing KB cells and measured transport of [^{3}H]vinblastine into vesicle space, which is ATP-dependent, and of much higher capacity (700 pmol/mg of protein), (8).

In agreement with previous studies we found the rank order of cytotoxics to displace [^{3}H]vinblastine binding to be vinblastine > vincristine > doxorubicin > colchicine (7). Quinidine is consistently found to be more potent than quinine to reverse the cytotoxic drug accumulation deficit of MDR cells (9), and accordingly we found quinidine to be 7-fold more potent than quinine to displace [^{3}H]vinblastine binding.

All four 1,4-dihydropyridines studied (see fig 2A and B) were found to greatly accelerate dissociation of [^{3}H]vinblastine from P-gp. Cyclosporin A, although a potent inhibitor of binding, did not (see Table I). As the K_D of a drug for a receptor is defined by the ratio of dissociation rate constant (k_{-1}units, min^{-1}) to association rate constant (k_{+1} units, $M^{-1}.min^{-1}$), then it is clear that K_D is proportional to k_{-1} (10). Theoretically a competitive inhibitor should not affect the dissociation rate constant (11) of another drug, as such an inhibitor can only bind when e.g. [^{3}H]vinblastine had dissociated from its binding site (which we have termed receptor site-1). As 1,4-dihydropyridines cause an increase in dissociation rate of [^{3}H]vinblastine from P-gp they must be acting at a site distinct from that which is vinblastine-selective, through a negative heterotropic allostric mechanism. The 1,4-dihydropyridine-selective site (receptor site-2) could be located on a protein other than P-gp, however as the 1,4-dihydropyridine [^{3}H]azidopine (12) and vinca alkaloid photoaffinity probes label P-gp (13), it is most likely that both the 1,4-dihydropyridine-selective and vinca-selective sites are located on P-gp. Clearly their binding domains must be topographically distinct, but allosterically coupled.

Of the cytotoxics we found that the anthracycline doxorubicin accelerated [^{3}H]vinblastine dissociation from P-gp 2-fold, whereas vincristine (a vinca alkaloid) etoposide (an epipodophylotoxin) and actinomycin D (a peptide) did not. This suggests that doxorubicin binds to a site distinct from the vinca alkaloid-selective site. We cannot determine if this action of doxorubicin is mediated through the 1,4-dihydropyridine-selective site, or a third drug binding site. The same argument applies to verapamil which caused a small but statistically significant 2-fold increase in dissociation rate of [^{3}H]vinblastine. This issue could be resolved by using a radioligand to bind to the 1,4-dihydropyridine-selective site directly.

In vesicles prepared from the Chinese hamster lung cell line DC-3F/ VCRd-5L it has been claimed that the 'binding' of a 1,4-dihydropyridine, [^{3}H]azidopine, to P-gp is inhibited allosterically by vinblastine (14). 'Binding' to vesicles was performed in transport buffer with ATP/Mg^{2+} . The 'B_{max}' was 4.5 nmol/mg protein, which, if the authors were measuring binding to P-gp with a molecular weight of 170 kDa would be equivalent to 70% of all membrane protein. However in MDR cells P-gp constitutes about 5% of vesicle protein (2). Evidently ATP-driven transport of [^{3}H]azidopine into vesicle space was measured which accounts for the extraordinarily high 'B_{max}' found (14).

Allosterically coupled drug binding domains at channels and pumps are not unusual and have been documented for the voltage-dependent sodium channel (15), the nicotinic acetylcholine receptor (16), the benzodiazepine/chloride ionophore (17), the NMDA receptor (18) and the voltage-dependent L-type calcium channel (19). In this paper we have demonstrated, using dissociation kinetics of [^{3}H]vinblastine, that P-gp harbours at least two distinct drug acceptor sites that are allosterically coupled. This finding has considerable importance for the description of the structure-activity relationships of drugs with activity at P-gp and may well explain some of the chemical heterogeneity of MDR-reversing agents.

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