Biochemical Pharmacology, Vol. 38, No. 18, pp. 3017–3027, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

DAUNORUBICIN AND VINCRISTINE BINDING TO PLASMA MEMBRANE VESICLES FROM DAUNORUBICIN-RESISTANT AND WILD TYPE EHRLICH ASCITES TUMOR CELLS

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(*Received* 3 *January* 1989; *accepted* 21 *March* 1989)

Abstract—Tumor cell resistance to anthracyclines, epipodophyllotoxins and vinca alkaloids, called multi-drug resistance (MDR) is intimately linked to changes in the plasma membrane which facilitate an increased energy dependent drug extrusion in the resistant cell compared to the wild type cell. Isolated plasma membrane vesicles from wild type Ehrlich ascites tumor cells (EHR2) and the daunorubicin (DNR) resistant subline EHR2/DNR+ were utilised to study binding and possible transport of DNR and vincristine (VCR). A significant ATP enhanced increase in VCR binding to vesicles from EHR2/DNR+ compared to EHR2 was demonstrated. Furthermore, an increase in ATP enhanced VCR binding in proportion to content of the MDR associated P-glycoprotein was seen in plasma membrane vesicles prepared from various benign human endocrine tumors. VCR binding to EHR2/DNR+ vesicles was inhibited by other vinca alkaloids > actinomycin D > colchicine > anthracyclines, with 35–75 μM concentrations of anthracyclines needed for 50% inhibition. VCR binding to EHR2/DNR+ vesicles was pH and temperature dependent with an activation energy of −30 kJ/mol and was decreased by replacement of Na^+ with K^+ and by addition of Ca^{2+}. Preincubation of vesicles with monoclonal antibody against the C terminal of P-glycoprotein had no effect on VCR binding and osmolality tests failed to show genuine transmembranal transport of VCR. DNR binding was similar in plasma membrane vesicles from both cell lines, and showed none of the characteristics mentioned for VCR. Furthermore, a radiolabeled *N*-hydroxysuccinimide ester derivative of doxorubicin, which inhibited VCR binding to EHR2/DNR+ membranes to an even greater extent than doxorubicin, labeled plasma membrane proteins from EHR2 and EHR2/DNR+ identically and did not demonstrate any binding to P-glycoprotein. Therefore, even though the study confirms the close link between vinca alkaloid binding and P-glycoprotein, it could not detect a similar association between anthracyclines and P-glycoprotein thus attesting to the complexity of the MDR phenotype.

The multi-drug resistance¶ (MDR) phenotype is the term applied to tumor cells which, after exposure *in vitro* or *in vivo* to cancer chemotherapeutic drugs such as anthracyclines, vinca alkaloids, epipodophyllotoxins, actinomycin D and colchicine not only develop resistance to the drug against which resistance is acquired, but also to the other, structurally unrelated, drugs. Furthermore, the MDR phenotype encompasses a decrease in intracellular drug concentration, the emergence of a 170 kDa plasma membrane glycoprotein termed P-glycoprotein and finally, the ability of a number of drugs such as calcium channel blockers, calmodulin inhibitors, lysosomotropic agents and detergents, in μM concentrations, to reverse resistance (reviewed in [1] and [2]). The decreased intracellular drug concentration in resistant cells has been found to be due to an increased energy dependent drug efflux together with a decrease in passive drug influx and a decrease in intracellular drug binding [3]. The mechanism of energy dependent drug efflux is still poorly understood though at least three theories have been advocated, namely, either that P-glycoprotein functions directly as an efflux pump for these diverse drugs [4], or as a pump for a drug carrier protein [5] or that the drugs are expelled via an exocytotic process which includes binding to P-glycoprotein [1].

To further elucidate the MDR phenotype, we have prepared plasma membrane vesicles from wild type and MDR cells and investigated the relationship between the two main classes of MDR drugs in binding to the plasma membrane under a variety of conditions.

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¶ Abbreviations used; ACLA, aclarubicin; ACT-D, actinomycin D: Ara-C, cytosine arabinoside, ATP, adenosine triphosphate, COL, colchicine; DNR, daunorubicin; DOX, doxorubicin; MOX, mitoxantrone; MDR, multi-drug resistance; MTX, methotrexate; NHSE-DOX, *N*-hydroxysuccinimide ester derivative of doxorubicin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; VBL, vinblastine; VCR, vincristine; VDS, vindesine.

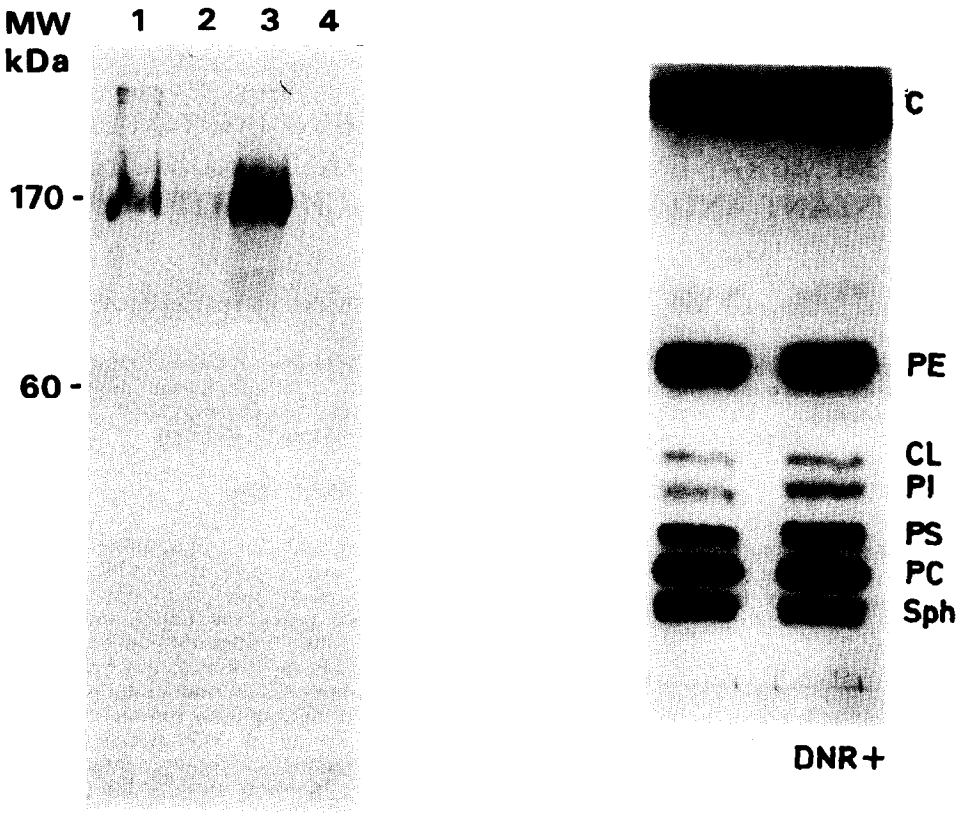


Fig. 1. (a) Western blot of plasma membranes from adrenal cortical adenoma (1), EHR2 (2), EHR2/DNR+ (3) and thyroid adenoma (4) probed with C219 antibody. (b) TLC of total lipid in plasma membrane preparations of EHR2 (left) and EHR2/DNR+ (right): C, cholesterol; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; Sph, sphingomyelin.

MATERIALS AND METHODS

Cell lines. The wild type Ehrlich ascites tumor EHR2 and its daunorubicin (DNR) resistant subline EHR2/DNR+ have previously been described in detail regarding cross resistance *in vivo* [6, 7] and drug uptake and efflux *in vitro* [3, 8, 9, 10]. Both sublines were maintained as ascitic tumors in first-generation hybrids of female Swiss mice and male inbred DBA/2 mice by weekly transplantation of 1.5×10^7 cells per mouse. Cells from the 7th day after transplantation were used for experiments. No drug was administered in the last transplantation before experiments.

In order to investigate drug binding in relation to P-glycoprotein in human tissue other than acquired MDR in *in vitro* cell lines, tissue from a typical benign adrenal cortical adenoma, a benign pheochromocytoma and a benign thyroid adenoma were processed to plasma membrane preparations as described for Ehrlich cells immediately after surgical removal.

Chemicals. [G^{3}H]DNR (1.9 Ci/mmol) was purchased from New England Nuclear (USA), and [G^{3}H] vincristine (8.2 Ci/mmol or 5.6 Ci/mmol in different batches), [14-^{14}C] doxorubicin (55 mCi/mmol) and *N*-succinimidyl [2,3-^{3}H]propionate (105 Ci/mmol) from Amersham (U.K.). Adenosine triphosphate (ATP), colchicine (COL), doxorubicin (DOX), vinblastine (VBL) and vincristine (VCR) were purchased from Sigma (St Louis, MO). DNR was obtained from Rhone-Poulenc (France), mitoxantrone (MOX) from Lederle (U.K.), actinomycin D (ACT-D) from Merck, Sharp and Dohme (U.S.A.), aclarubicin (ACLA) from Lundbeck (Denmark), vindesine (VDS) from Lilly (U.S.A.), methotrexate (MTX) from Pharmacia (Denmark), cytosine arabinoside (Ara-C) from Upjohn (Belgium) and verapamil (VER) from Knoll (F.R.G.).

Plasma membrane isolation. The primary plasma membrane vesicle isolation procedure was developed in our laboratory based on experiences from isolation of kidney brush border membrane vesicles [11] with further purification on discontinuous Ficoll gradients according to Spitzer *et al.* [12]. Approximately 5–7×10^9 cells from a pool of roughly 10 animals or 5–10 g of endocrine tumor tissue were processed in each batch. All steps were performed either on ice or in systems cooled to under 5°. Cells were first washed four times in ice-cold Ringer's solution containing 148 mM Na^+, 5.2 mM K^+, 151 mM Cl^-, 1.7 mM Ca^{2+}, 1.2 mM Mg^{2+}, 1.2 mM SO_4^- and 3.0 mM orthophosphate (pH 7.45), and hereafter spun gently at 110 *g* for 3 min whereby a cell sediment was formed. Aliquots of 0.5 ml cell sediment were diluted in 10 ml homogenisation buffer (pH 7.4) consisting of 10 mM histidine, 0.5 mM EDTA, 0.5 mM

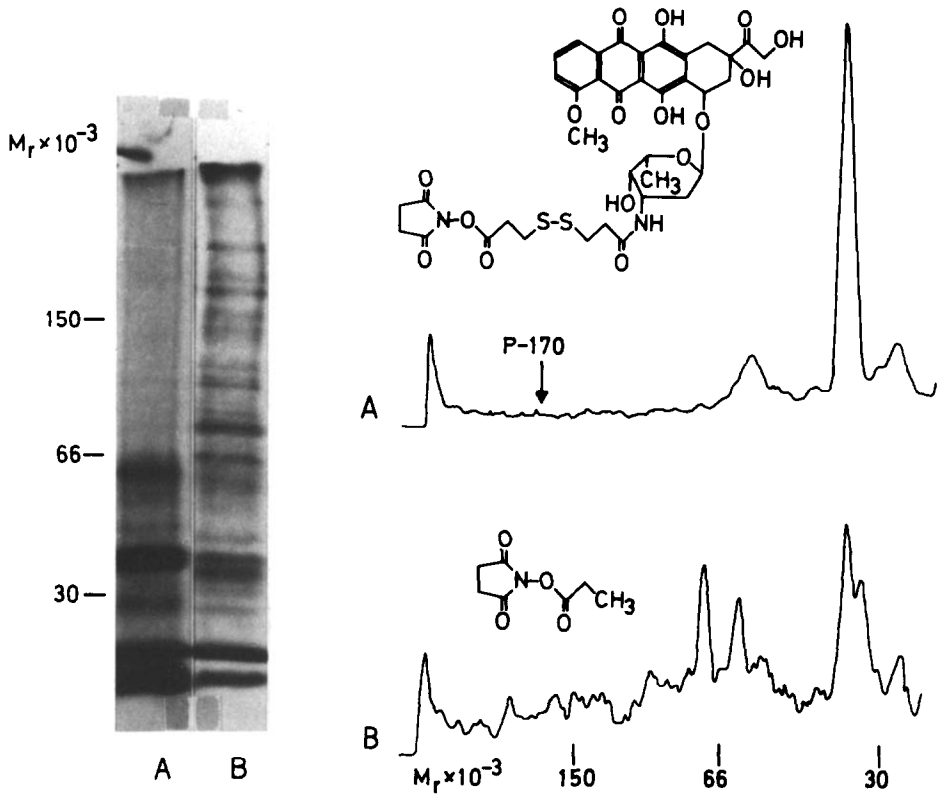


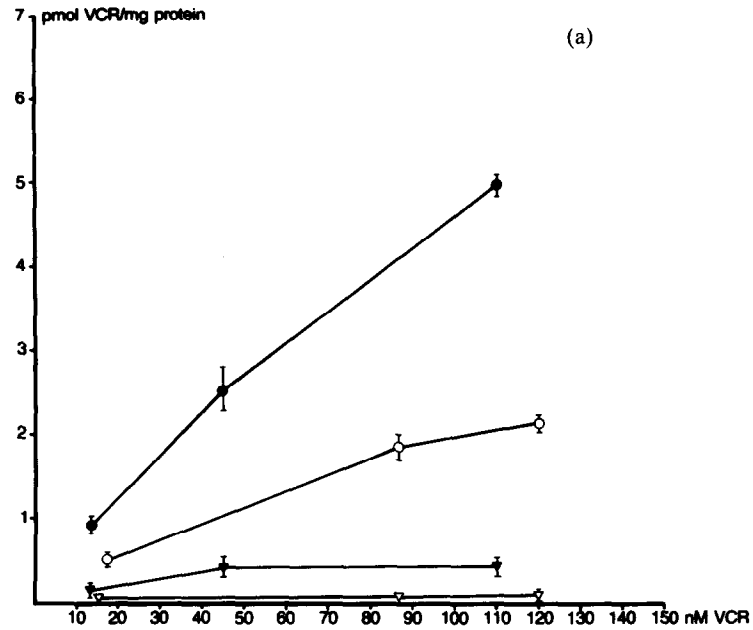
Fig. 2. Photodensitometric scans of fluorographs obtained after labeling of proteins in plasma membranes from EHR2/DNR+ with either N-hydroxysuccinimide ester derivative of [14-^{14}C]DOX (0.6 nmol/mg protein) (A), or with *N*-succinimidyl [2,3-^{3}H]propionate (0.13 nmol/mg protein) (B). Separation of proteins was by SDS–PAGE as described in materials and methods. The dense fluorographic bands with $M_r < 30$ kDa represent labeled membrane lipids and excess of unreacted labeling reagents. The position of P-glycoprotein in the gels is indicated as P-170.

dithiothreitol and 0.1 mM phenylmethylsulphonyl fluoride, and cells allowed to swell for 15 min. Hereafter cells were disrupted by polytronation (setting 3 for 30 sec). $ZnCl_2$ 10 mM, 10% v/v, (pH 7.2) was added and the homogenate stirred for 15 min before centrifugation at 1,400 *g* for 10 min. The supernatant (S1) was then centrifuged at 48,000 *g* for 30 min and the resulting pellet (P1) resuspended in homogenisation buffer to a final volume of 60 ml after repeated passages through an Ependorff 5 ml pipette tip. This solution was again centrifuged at 48,000 *g* for 30 min. The resulting pellet (P2) was again passaged through a pipette tip and diluted in homogenisation buffer to a final volume of 4 ml, which was then layered on top of a discontinuous Ficoll/sucrose gradient exactly as described in [12]. After ultracentrifugation at 100,000 *g* in a Beckmann SW28 rotor for 60 min, the plasma membrane fraction was collected from the interface between the homogenisation buffer and the 3% Ficoll/sucrose layer and resuspended in a 0.3 M sucrose solution, containing 20 mM Hepes (pH 7.4). After a second ultracentrifugation at 100,000 *g* for 30 min, the resulting pellet was resuspended in the same 0.3 M sucrose/20 mM Hepes solution, calibrated to a protein concentration of 5 mg/ml and stored in 0.25 ml aliquots at −80° for up to 3 weeks.

Enzyme assays. The following assays were used to determine enrichment and recovery of cellular organelles in the plasma membrane fraction in relation to homogenate. Plasma membrane: ouabain sensitive ($Na^+ - K^+$)-ATPase [13], Endosomes: Leucyl-beta-naphthylamidase [14]; Endoplasmic reticulum: glucose-6-phosphatase [15]; Lysosomes: acid phosphatase [16]; Mitochondria: succinate dehydrogenase [17]; Golgi complex: thiamine pyrophosphatase [18]. Protein determination was performed with the BioRad assay system (Biorad Laboratories, Munich, F.R.G.) with bovine plasma gammaglobulin as standard.

Electron microscopy. Aliquots (0.25 ml) of plasma membrane preparation were fixed in equal volumes of a 70% dilution of Karnovsky's fixative [19] overnight at 4° and thereafter treated as previously described [20].

Lipid analysis. Total lipid extracts were obtained by chloroform/methanol extraction of isolated membranes as described in [21]. Separation of individual lipids was performed by TLC on 0.25 mm silica gel 60 plates (Merck, Darmstadt, F.R.G.) in the solvent system chloroform/methanol/2-propanol/0.25% KCl/ethyl acetate (30:9:25:6:18, v/v) [22], and quantitative analysis of the lipid components was carried out using photodensitometry after charring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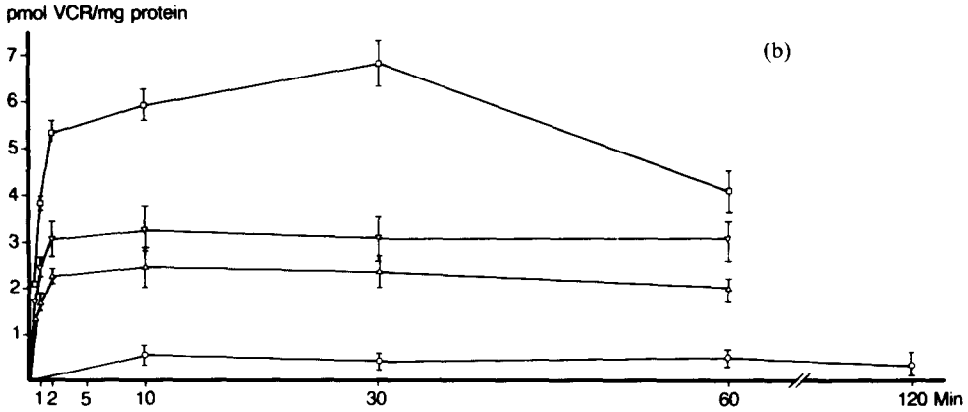
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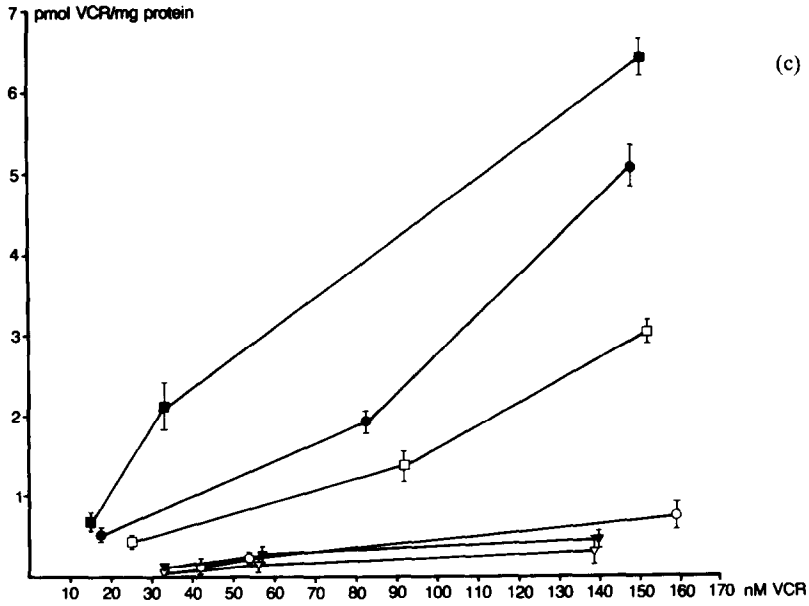
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the plates with 3% $CuSO_4$/8% H_3PO_4 at 180°. Two-dimensional TLC was carried out using the solvent system chloroform/methanol/concentrated NH_3 (74:36:5, v/v) (first dimension) and chloroform/methanol/acetic acid (46:15:6, v/v) (second dimension).

Immunological detection of P-glycoprotein. C219 monoclonal antibody directed against the C terminal end of P-glycoprotein [23] was purchased from Centocor, Malvern PA (U.S.A.) and peroxidase conjugated rabbit anti-mouse antibody (Dako P260) from Dakopatts (Denmark).

Aliquots (50 μl) of plasma membrane preparations (5 mg/ml) were made 1% in SDS and loaded onto 10% SDS–PAGE gels with 0.1% SDS [24]. Following electrophoresis, proteins were transferred to nitrocellulose paper [25]. The paper was incubated in 150 mM NaCl, 50 mM Tris buffer [pH 7.4] containing 3% bovine serum albumin and 0.1% v/v Tween 20, and probed overnight at 4° with the C219 antibody at 1:400 dilution. Peroxidase conjugated rabbit anti-mouse antibody was used as the secondary antibody (dilution 1:250, 2 hr), and the blots were developed using 3-amino-9-ethyl carbazole as chromogen. Controls were performed by omission of the primary antibody.

Synthesis of NHSE-DOX. A radiolabeled DOX derivative (NHSE-DOX) containing a reactive *N*-hydroxysuccinimide ester moiety [26] was synthesized by reaction of 90 nmol [14-^{14}C]DOX with a 10-fold molar excess of the bifunctional reagent dithiobis(succinimidyl propionate) [27]. The derivative was purified by column chromatography on silica gel 60 with a yield of 55 nmol (60%). Purity was 95% by TLC (chloroform/methanol/acetic acid, 30:5:2.5, R_f 0.83.).

Protein labeling in plasma membranes by NHSE-DOX. Plasma membrane vesicles (800 μg protein) in 100 μl of 20 mM Na/Hepes buffer (pH 7.4) were incubated for 2 hr at 25° with either *N*-succinimidyl [2,3-^{3}H]propionate (0.1 nmol, 10 μCi) or NHSE-[14-^{14}C]DOX (0.6 nmol, 0.028 μCi) added in 5 μl of dimethylsulfoxide. After the incubation, 50 μl aliquots were made 1% in SDS and analyzed by SDS–PAGE using 10% gels with 0.1% SDS [24]. Following electrophoresis, gels were stained for protein with Coomassie brilliant blue, and then prepared for fluorography in Amplify™ (Amersham International, U.K.). Fluorographs were obtained with Kodak X-Omat film exposed for 12 days at −80°.

Determination of drug binding to plasma membrane vesicles. Drug binding was assayed with tracers using rapid mixing and filtration [11]. Plasma membrane (10 μl) preparation with a protein concentration of 5 mg/ml and 20 μl incubation buffer containing tracer were pipetted in two separate drops at the lower sides of a plastic test tube. The experiment was initiated by vortexing the test tube for 6 sec at a preset speed and transfer to a 37° water bath (all reactions were carried out at 37° except when otherwise stated) for the desired time period. The experiment was terminated by the addition of 0.8 ml ice-cold 150 mM NaCl, 10 mM Tris/Hepes, pH 7.4. This solution was then immediately filtered, under light suction, through a 2.5 cm in diameter Whatman GF/F glassfiber filter presoaked in 20% fetal calf serum. The filter was then rinsed twice with 4 ml ice-cold NaCl–Tris/Hepes, dried in an oven at 55° for approximately 30 min and subsequently covered with 8 ml Packard Opti-fluor liquid scintillation solution and counted. All experimental points were done in triplicate. Background values were of two kinds and were measured for each experiment: (a) the amount of tracer retained by the filter alone, i.e. when no plasma membrane preparation was present, and (b) the amount of tracer retained on the filter when the experiment had been terminated by addition of 0.8 ml ice-cold NaCl–Tris/Hepes simultaneously with the vortexing of the plasma membrane preparation with the reagent solution and subsequent immediate filtration and washes, i.e. zero time binding. This latter background value is hereafter termed non-specific membrane binding. Except when otherwise specified, incubation buffer contained 0.2 M sucrose, 50 mM NaCl and 10 mM Tris/Hepes, pH 7.4. In experiments utilising ATP, 5 mM ATP together with 5 mM $MgCl_2$ was used in the incubation buffer giving a final experimental concentration of 3.3 mM ATP and $MgCl_2$. Alteration of sucrose concentrations were used to change osmolality in the osmolality tests.

All experiments were performed a minimum of three times utilizing at least two different membrane batches.

RESULTS

Characterisation of plasma membrane preparations

The results of the marker enzyme assays (mean ± SD of 3 determinations) confirmed those of Spitzer *et al.* [12] in that the plasma membrane was enriched 22-fold (±4) with a recovery of 11% (± 2.5) relative to homogenate. Endosomes were also greatly enriched, namely 15-fold (±3) with a recovery of 4.5% (± 0.8). Contamination by other organelles was reasonably low, with enrichments of 0.7 (±0.2)

Fig. 3. (a) Dose–response curves of [^{3}H]VCR binding to plasma membrane vesicles from EHR2 and EHR2/DNR+ sublines. All experiments performed at 37° and 10 min. Bars = SD of 3 determinations. ▼——▼, EHR2 + ATP; ▽——▽, EHR 2 − ATP, ●——●, EHR2/DNR+ + ATP; ○——○, EHR2/DNR − ATP. (b) Time course of [^{3}H]VCR binding to EHR2/DNR+ plasma membrane vesicles in the presence of ATP. All experiments at 37° except when specified. VCR concentration was 150 nM. □——□, Standard incubation medium; ▽——▽, Incubation medium with total replacement of Na^+ with K^+; △——△, Standard incubation medium with addition of 2 mM $CaCl_2$; ○——○, binding at 4° in standard incubation medium. (c) Dose–response curves of [^{3}H]VCR binding to plasma membrane vesicles from adrenal cortical adenoma, pheochromocytoma and thyroid adenoma: ■——■, = adrenal cortical adenoma + ATP; □——□, = adrenal cortical adenoma − ATP; ●——●, pheochromocytoma + ATP; ○——○, = pheochromocytoma − ATP; ▼——▼, = thyroid adenoma + ATP; ▽——▽, = thyroid adenoma − ATP.

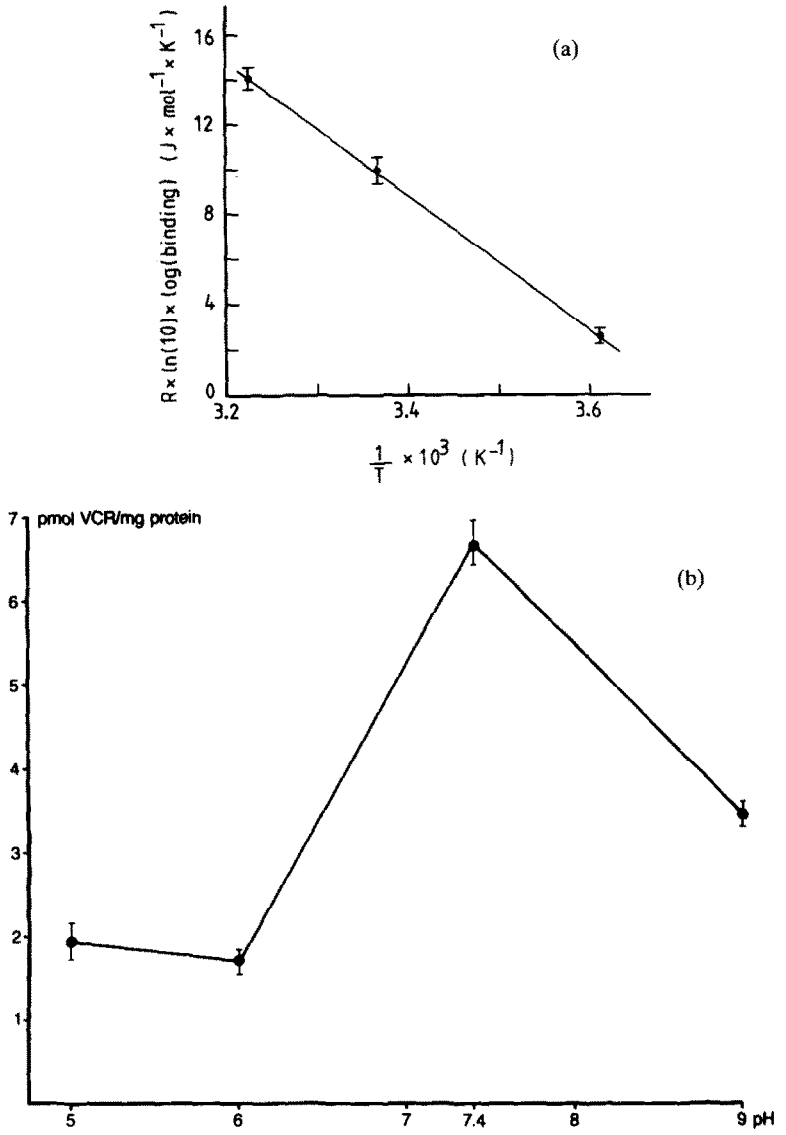


Fig. 4. (a) Arrhenius plot of the temperature dependency of [^{3}H]VCR binding to EHR2/DNR+ plasma membrane vesicles in medium containing ATP. Binding was measured at 4, 24 and 37°. VCR concentration was 135 nM. (b) [^{3}H]VCR binding to EHR2/DNR+ plasma membrane vesicles with varying pH of the incubation medium. Medium contained ATP. VCR concentration was 150 nM.

for lysosomes, 0.3 (±0.05) for Golgi complex, 4.4 (±1.6) for endoplasmic reticulum and 1.7 (±1.1) for mitochondria, and recoveries of less than 2% for these organelles. Electron microscopy showed that the great majority of the preparation consisted of smooth vesicles with diameters ranging from 0.2 to 0.7 μm. In addition, rare multivesicular bodies, lysosomes and a few mitochondria were encountered, thus confirming the enzymatic analysis. Lipid analysis as well as Coomassie stained SDS gels showed no significant difference between plasma membrane preparations from EHR2 and EHR2/DNR+ (Fig. 1). The lipid profile is consistent with that of the plasma membrane with some minor mitochondrial contamination as evidenced by the presence of small amounts of cardiolipin (Fig. 1). Furthermore, both lipid and protein analysis on different batches of plasma membranes showed identical patterns indicating negligable inter batch variation. Immunoblots showed the emergence of P-glycoprotein in EHR2/DNR+ and in the adrenal cortical adenoma but not in EHR2 or in the thyroid adenoma (Fig. 1). Unfortunately, insufficient material was available for immunoblot of plasma membrane preparations from the pheochromocytoma. However, immunoperoxidase staining with C219 antibody on frozen sections using a previously described methodology [28], from all three adenomas showed heavy staining of plasma membranes in the adrenal cortical adenoma, light to moderate staining in the pheochromocytoma and no staining in the thyroid adenoma (not shown).

Labeling of proteins in plasma membranes by NHSE-DOX

To identify anthracycline-binding membrane proteins, EHR2 and EHR2/DNR+ plasma membranes were incubated with a radiolabeled derivative of DOX containing a reactive *N*-hydroxysuccinimide easter group (Fig. 2). This derivative reacts readily with free amino groups in proteins, whereby the anthracycline is linked to the surface of the proteins via stable amide bonds. Protein binding of DOX was determined by fluorography following separation of proteins by SDS–PAGE. Results with EHR2/DNR+ membranes are shown in Fig. 2. A 34 kDa protein was the most prominently labeled, and no significant labeling at the position of the P-glycoprotein in the 170 kDa region was found. When labeling was carried out with a *N*-hydroxysuccinimide ester (*N*-succinimidylpropionate) without the DOX moiety, the labeling pattern followed the Coomassie blue stained protein pattern (Fig. 2). No difference between labeling patterns in EHR2 and EHR2/DNR+ plasma membranes was detected (not shown).

VCR binding to plasma membrane vesicles

VCR demonstrated only low (<10%) non-specific membrane binding values compared to experimental values. Plasma membranes from EHR2/DNR+ bound significantly more VCR in a dose dependent manner compared to plasma membranes from EHR2 (Fig. 3a). Furthermore, this difference was greatly augmented in the presence of ATP. The time course of VCR uptake was rapid, being complete within the first ten minutes with a slow decline in values after 30 min (Fig. 3b), therefore a 10 min incubation was chosen as the standard in all experiments. Total replacement of sodium with potassium or addition of 2 mM Ca^{2+} both resulted in decreased uptake. Plasma membranes isolated from the human benign endocrine adenomas demonstrated the same ATP enhanced VCR binding which correlated with their content of P-glycoprotein, i.e. adrenal cortical adenoma > pheochromocytoma > thyroid adenoma (Fig. 3c).

The temperature dependency of VCR binding to EHR2/DNR+ plasma membrane vesicles is shown in Fig. 4(a). The activation energy, E_a, was calculated to be -29.3 ± 3.4 kJ/mol by the formula: Binding = constant $\times \exp(E_a/RT)$, where R is the gas constant and T is the temperature in Kelvin. Prolonged incubation at 4° (up to 2 hr, Fig. 3b) showed no further binding after 10 min.

VCR binding to EHR2/DNR+ vesicles was sensitive to variations of pH in the incubation medium as demonstrated in Fig. 4(b). However incubation in 10 mM NH_4Cl did not influence binding, signifying that binding was not due to a transvesicular proton gradient.

Osmolality experiments were performed on plasma membranes from both EHR2/DNR+ and the pheochromocytoma with osmolalities varying from 0.1 to 1.0 Osm. No osmotic dependence of VCR binding was evident (not shown).

Experiments were performed with preincubation of membrane vesicles from EHR2/DNR+ at 4° for 30 min with varying concentrations of C219 antibody from 0.05 μg/50 μl membrane vesicles to 5 μg/50 μl membrane vesicles and subsequent addition of VCR containing medium and transfer to 37° for 10 min. No effect on VCR binding by C219 was detected (results not shown).

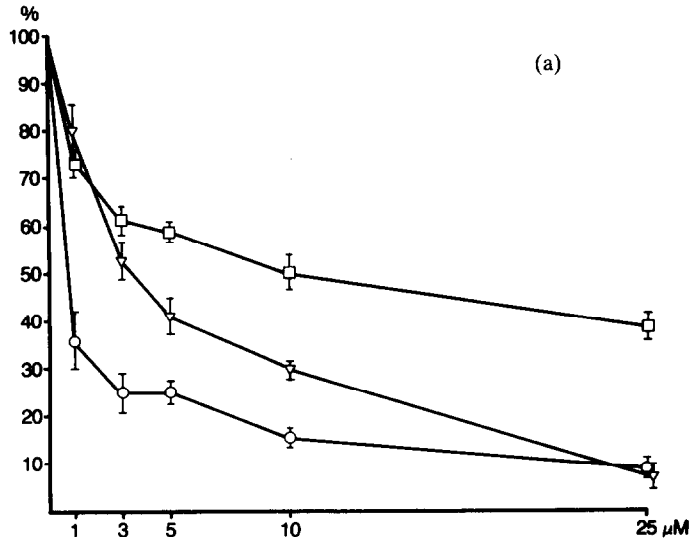
Incubation of EHR2/DNR+ vesicles with drugs belonging to the MDR family showed that vinca alkaloids inhibited VCR binding to EHR2/DNR+ vesicles by 50% in the low (1–10 μM) range (Fig. 5a), ACT-D and COL in the intermediate (20–30 μM) range (Fig. 5b) and anthracyclines in the high range (35–75 μM) (Fig. 5c), while Ara-C and MTX, which are not MDR drugs, were without effect in concentrations up to 100 μM (Fig. 5b).

DNR binding to plasma membrane vesicles

No difference in DNR binding to plasma membrane preparations from EHR2 and EHR2/DNR+ was detected. High-affinity binding of anthracycline to the negatively charged membrane lipid cardiolipin has been reported with an association constant of $1.6 \times 10^6\ M^{-1}$ [29]. Our membrane preparations contained about 10 nmol cardiolipin per mg protein (Fig. 1). However, DNR appeared only to bind to low affinity sites in the membranes and increased linearly with DNR concentration up to 3.5 μM when 0.3 nmol DNR was bound per mg protein. Addition of ATP to the medium made no difference. The binding of DNR to membranes was without time or temperature dependency as the non-specific membrane binding values (see Materials and Methods for definition) for each concentration were identical to the 10 min 37° values. The amount of isotope retained by the filter alone was less than 10% of that bound by plasma membranes. Manipulation of salt and buffer strengths did not have any effect on the immediate adherence of DNR to membranes. Neither simultaneous nor 30 min preincubations at 4° with VCR (25 μM) had any effect on DNR binding.

DISCUSSION

The MDR phenotype is intimately linked with P-glucoprotein as transfection studies have demonstrated that the MDR1 gene, which is sufficient for the conferral of MDR, codes for P-glycoprotein [30, 31]. Furthermore, homologies exist between P-glycoprotein and bacterial transport proteins [5, 32, 33]. It is therefore very probable that P-glycoprotein is responsible for the decrease in drug accumulation which is the hallmark of MDR. However, the actual mechanism whereby P-glycoprotein accomplishes this is still unsettled [1, 2]. The present study has demonstrated that highly purified plasma membrane vesicles from MDR cells bind more VCR than plasma membranes from sensitive cells in a dose, temperature and pH dependent manner, and that ATP in the presence of $MgCl_2$ greatly enhances this difference in drug binding. These results are similar to those of Cornwell *et al.* [34] and Horio *et al.* [35] on VBL binding to plasma membrane vesicles from human KB cells and to results in a recent paper by Naito *et al.* [36] on VCR binding in K562 cell lines. Together with results from photoaffinity labeling studies on vinca alkaloid analogues [37, 38], there can thus be little doubt that vinca alkaloids bind to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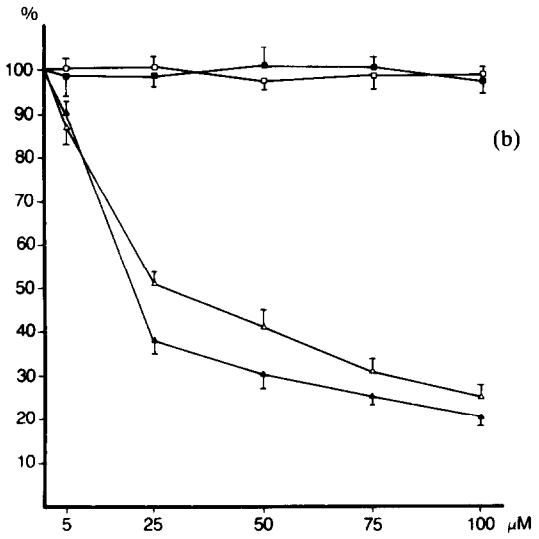
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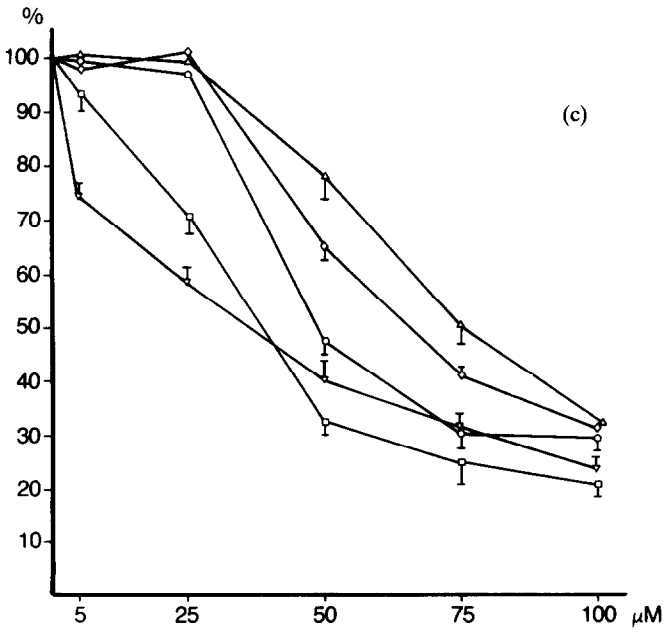
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75
100 μM

P-glycoprotein in plasma membrane vesicles from MDR cells and that this binding is enhanced by the presence of ATP with $MgCl_2$. That the binding of VCR to P-glycoprotein is specific is supported by inhibition with analogues shown in Fig. 5(a) where VBL, which only differs from VCR at one site in the molecule, is a better inhibitor than VDS which differs from VCR at three sites. Furthermore, we have shown that ATP enhanced VCR binding is present in benign human endocrine adenomas, i.e. tumors which have never been treated with MDR drugs, in relation to their content of P-glycoprotein. This implies that the P-glycoprotein which has been immunologically detected in normal human tissue [39] has similar drug binding capacities to the P-glycoprotein described in acquired experimental resistance.

The decreased binding of VCR to EHR2/DNR+ plasma membranes in the presence of 2 mM Ca^{2+} is corroborated by the finding of Hamada *et al.* that Ca^{2+} in millimolar concentrations inhibited the Mg^{2+}-catalyzed hydrolysis of ATP by P-glycoprotein [40]. They did not find an inhibitory effect of K^+ though this could be due to their use of a lower K^+ concentration (20 mM) than in the present study (50 mM).

Osmotic dependence is an indicator of a genuine transmembrane transport process [41]. Two previous studies which have dealt with osmotic tests on vinca alkaloid binding to MDR plasma membrane vesicles reached different results. Thus, Horio *et al.* showed osmotic dependency of VBL uptake in plasma membrane vesicles from MDR KB-V1 cells [35], while Cornwell *et al.* did not find osmotic dependency on VBL binding to vesicles from MDR KB-C4 cells [34]. We were not able to demonstrate osmotic dependency of VCR binding to EHR2/DNR+ plasma membrane vesicles and thus did not show a P-glycoprotein "pump". This is in accordance with the low activation energy around −30 kJ/mol (= −7 kcal/mol) which would indicate that association of VCR with EHR2/DNR+ plasma membrane vesicles is either a simple adsorption or a passage through hydrophilic pores [42]. Penetration of the plasma membrane either by dissolving into the hydrocarbon phase [43], by transfer via facilitated diffusion [44] or by active transport associated with ATPase activity [45] usually have activation energies in excess of −60 kJ/mol and exhibit a phase transition not seen for VCR (Fig. 4a). An important result of the present study is therefore that an ATP enhanced increase in VCR binding to MDR plasma membrane vesicles can take place without evidence of transport into the vesicles. The most logical explanation for this is that ATP induces a conformational change in P-glycoprotein which in turn leads to a greater binding capacity for VCR.

There are interesting differences between the reported inhibition by MDR drugs of vinca alkaloid binding to plasma membrane vesicles from various resistant lines. Thus, while both our study and the studies on KB-V1 [35] and K562/DOX [36] cell lines show 50% ACT-D inhibitions in the 5–25 μM range, the report on KB-C4 cells demonstrated a 50% ACT-D inhibition of 100 μM [34]. Furthermore, none of the other studies have shown 50% inhibition by COL under 100 μM [34, 35, 36], while we found a 50% inhibition by COL of 25–30 μM (Fig. 5b). These differences could be explained by diversities in the structure of the P-glycoprotein molecule in different MDR cell lines and support the notion of a P-glycoprotein family [2]. However, inhibition of vinca alkaloid binding by various anthracyclines is remarkably similar with 50% inhibitions reported to be from 30–60 μM [34, 35, 36], and 50% inhibition in our study was accomplished in the 35–75 μM range for the four anthracyclines tested (Fig. 5c).

Though ATP enhanced VCR and VBL binding to MDR plasma membranes is thus well established, corresponding studies on anthracycline binding have not previously been reported. The above mentioned inhibition of VBL and VCR binding to MDR plasma membrane vesicles by anthracyclines has been construed as a specific anthracycline binding to P-glycoprotein [35, 36]. However, multiple effects in the plasma membrane have been observed to be caused by anthracycline concentrations as high as 30–75 μM [46, 47, 48], in particular changes in fluidity are almost certain to occur [49], changes which could in turn influence VCR and VBL binding to P-glycoprotein. In this context it is notable that ACLA, which is by far the most lipophilic of the four anthracyclines shown in Fig. 5(c) is also the anthracycline (apart from NHSE-DOX) which shows the earliest and greatest inhibition of VCR binding. In addition, while DOX has an 1-octanol/water phase partition coefficient of approximately 1, NHSE-DOX is much more lipophilic with a coefficient of 200 and is also a considerably better inhibitor of VCR binding than DOX itself (Fig. 5c). Several other observations do not favour a specific binding of anthracyclines to P-glycoprotein: (1) covalent labeling of plasma membrane proteins with NHSE-DOX demonstrated no significant labeling of the 170 kDa range in SDS–PAGE fluorographs (Fig. 2) even though it inhibited VCR binding more efficiently than the other anthracyclines. (2) The single reported photoaffinity labeling study with an anthracycline analogue on MDR cells made no mention of labeling seen in the 170 kDa region [50]. (3) Studies on both K562 and 2780 MDR cells using the MRK-16 monoclonal antibody directed against an extracellular epitope of P-glycoprotein showed increased accumulation of VCR by MRK-16 but no effect on DNR accumulation [51, 52]. (4) We were not able to demonstrate any difference in DNR binding to plasma membrane vesicles from MDR and sensitive cells and (5) no effect of VCR was found on DNR binding to MDR

Fig. 5. Inhibition of [^{3}H]VCR binding to EHR2/DNR+ plasma membrane vesicles in medium containing ATP. VCR concentration was 130–145 nM. (a) ○——○, unlabeled VCR; ▽——▽, VBL; □——□, VDS. (b) ▲——▲, ACT-D; △——△, COl; ■——■, MTX; □——□, Ara-C. (c) △——△, = MOX; ◇——◇, = DOX; ▽——▽, NHSE-DOX; ○——○, = DNR; □——□, = ACLA.

membranes. We conclude that it appears that vinca alkaloids and anthracyclines differ significantly with respect to binding to P-glycoprotein. We believe that inhibition of VCR binding to P-glycoprotein by anthracyclines is not due to specific interaction at a VCR binding locus on P-glycoprotein but is instead presumably caused by conformational changes in P-glycoprotein secondary to anthracycline perturbation of the lipid bilayer, either caused directly or by interaction with the 34 kDa protein demonstrated in Fig. 2.

It is usually assumed that the energy dependent efflux mechanism of MDR constitutes a single common pathway, and at least three hypotheses have been put forward, namely (1) that P-glycoprotein acts as a pump molecule per se for all the different drugs of the MDR family [4], (2) that P-glycoprotein acts as a pump for a drug carrier protein analogous to the haemolysin transporter in *E. coli* or the membrane-bound components of the hisP and malK bacterial periplasmic transport systems [5] and (3) that P-glycoprotein regulates vesicular membrane traffic by which process MDR drugs would be exocytosed from the cell [1, 53, 54]. Though the results on vinca alkaloids would be consistent with the first hypothesis, it is difficult, based on the above mentioned considerations, to fit anthracycline transport together with a "pure" P-glycoprotein pump. The second hypothesis is attractive from both functional and structural aspects, however no drug carrier protein has yet been identified. The third hypothesis is also a possibility, however the regulation of vesicular traffic by P-glycoprotein remains to be elucidated. Finally, it is conceivable that the decrease in intracellular accumulation of the different drugs in MDR cells is caused by different mechanisms (though all associated with P-glycoprotein) leading to the same result, e.g. that vinca alkaloids are pumped by P-glycoprotein while anthracyclines are exocytosed. In support of different mechanisms behind the MDR phenotype is evidence associating COL resistance with decreased uptake instead of increased efflux [2].

Acknowledgements—The expert technical assistance of Marianne Knudsen, Vibeke Sejer, Maria Andersen and Jytte Amtorp is gratefully acknowledged. Supported by the Danish Cancer Society.

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