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Characterisation of Multidrug-Resistant Ehrlich Ascites Tumour Cells Selected *In Vivo* for Resistance to Etoposide

Dorte Nielsen,† Christian Maare,* Jens Eriksen,* Thomas Litman,* Ellen Friche‡ and Torben Skovsgaard**

*Department of Oncology, Herlev Hospital, University of Copenhagen, Copenhagen and ‡Department of Haematology, Finsen Centret, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

ABSTRACT. An Ehrlich ascites tumour cell line (EHR2) was selected for resistance to etoposide (VP16) by *in vivo* exposure to this agent. The resulting cell line (EHR2/VP16) was 114.3-, 5.7-, and 4.0-fold resistant to VP16, daunorubicin, and vincristine, respectively. The amount of salt-extractable immunoreactive topoisomerase IIα and β in EHR2/VP16 was reduced by 30–40% relative to that in EHR2. The multidrug resistance-associated protein (MRP) mRNA was increased 20-fold in EHR2/VP16 as compared with EHR2, whereas the expression of P-glycoprotein was unchanged. In EHR2/VP16, the steady-state accumulation of [^{3}H]VP16 and daunorubicin was reduced by 64% and 17%, respectively, as compared with EHR2. Deprivation of energy by addition of sodium azide increased the accumulation of both drugs to the level of sensitive cells. When glycolysis was restored by the addition of glucose to EHR2/VP16 cells loaded with drug in the presence of sodium azide, extrusion of [^{3}H]VP16 and daunorubicin was induced. Addition of verapamil (25 μM) decreased the efflux of daunorubicin to the level of sensitive cells, but had only a moderate effect on the efflux of [^{3}H]VP16. The resistant cells showed moderate sensitisation to VP16 on treatment with verapamil, whereas cyclosporin A had no effect. Compared with that of sensitive cells, the ATPase activity of plasma membrane vesicles prepared from EHR2/VP16 cells was very low. Vanadate inhibited the ATPase activity of EHR2/VP16 microsomes with a K_i value of 30 μM. ATPase activity was slightly stimulated by daunorubicin, whereas vinblastine, verapamil, and cyclosporin A had no effect. In conclusion, development of resistance to VP16 in EHR2 is accompanied by a significant reduction in topoisomerase II (α and β) and by increased expression of MRP mRNA (20-fold). MRP displays several points of resemblance to P-glycoprotein in its mode of action: 1) like P-glycoprotein, MRP causes resistance to a range of hydrophobic drugs; 2) MRP decreases drug accumulation in the cells and this decrease is abolished by omission of energy; and 3) MRP increases efflux of drug from cells. However, compared with that of P-glycoprotein-positive cells, the ATPase activity of MRP-positive cells is found to be low and not able to be stimulated by verapamil. BIOCHEM PHARMACOL **60**;3:353–361, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. drug resistance; etoposide; multidrug resistance-associated protein; topoisomerase II; tumour cell lines

Resistance to multiple drugs is one of the major limitations to the successful treatment of many human cancers. Drugs such as anthracyclines and epipodophyllotoxins are widely used as antitumour agents. Two main categories of resistance mechanisms are currently known to interfere with this kind of drug. The first concerns mechanisms that result in a reduced drug concentration at the target site and bring about an MDR§ phenotype. This phenotype is known to be conferred by at least two proteins: P-gp, encoded by the *mdr1* gene [1], and the more recently identified MRP [2]. Both P-gp and MRP belong to the ATP-binding cassette superfamily of transport proteins [1, 2]. The MDR proteins are, however, structurally different and share only a limited sequence identity in regions involving nucleotide-binding domains [2]. MRP is a 190-kDa integral membrane phosphoglycoprotein encoded by a 6.5-kb mRNA [2, 3]. The cDNA encoding MRP was originally cloned from an MDR small cell lung cancer cell line [2, 4] and has subsequently been found to be expressed in a number of drug-selected cell lines [5–9]. The drugs most often involved in MRP-mediated resistance are the anthracyclines [5, 6], but the resistance mechanism has also been reported after selection with VCR [7] and VP16 [8, 9].

The second category of resistance involves alterations

† Corresponding author: Dr. Dorte Nielsen, Dept. of Oncology R, University of Copenhagen, Herlev Hospital, Herlev Ringvej, DK-2730 Herlev, Denmark. Tel. +45 448 84 714; FAX +45 448 83 094; E-mail: dln@dadlnet.dk

§ *Abbreviations:* CsA, cyclosporin A; DNR, daunorubicin; EHR2, Ehrlich ascites tumour cell line; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; PCR, polymerase chain reaction; P-gp, P-glycoprotein; RT–PCR, reverse transcriptase–polymerase chain reaction; Topo II, topoisomerase II; VBL, vinblastine; VCR, vincristine; VER, verapamil; and VP16, etoposide.

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affecting the drug target, the enzyme Topo II. DNA Topo II is a proliferation-dependent protein that requires ATP to relax DNA molecules. It plays an essential role in chromatin assembly, chromosome condensation, and decondensation [10]. Two isoforms of Topo II have been demonstrated. The smaller 170-kDa form is termed Topo IIα and the larger 180-kDa form Topo IIβ. These have different biochemical and pharmacological profiles [11].

In the present study, we examined the resistance mechanisms and the kinetics of VP16 and DNR transport in a VP16-selected tumour cell line.

MATERIALS AND METHODS

Drugs and Chemicals

VP16 was obtained from Bristol-Myers Squibb, VCR and VBL from Eli Lilly and Co., and DNR from Pharmacia Upjohn. [^{3}H]VP16 was obtained from Moravek Biochemicals. Sodium azide was provided by Merck, VER by Ercopharm Ltd., and CsA by Sandoz. Agarose and Bio-Rad dye reagent were purchased from Bio-Rad Laboratories. The protease inhibitors were from Sigma Chemical Co. PCR buffer, avian myeloblastosis virus (AMV) reverse transcriptase, Taq DNA polymerase, and random hexadeoxynucleotide primers were obtained from Boehringer Mannheim. Deoxynucleotides and RNAguard ribonuclease inhibitor were from Pharmacia, and primers from DNA Technology.

Tumour Cell Lines

The sensitive EHR2 cell line and a VP16-resistant subline (EHR2/VP16) were used for the experiments. The resistant subline (EHR2/VP16) was developed and maintained *in vivo* in mice by intraperitoneal treatment with VP16 20 mg/kg × 4 weekly, corresponding to LD10. Cell lines were frozen (passage 173) and all experiments performed within a few passages to avoid experimental variation owing to evolution of the tumour cells.

Clonogenic Assay

The cytotoxicity of VP16, DNR, or VCR was measured by the clonogenic assay [12]. IC_{50} values were defined as the drug concentrations inhibiting 50% of colony formation. The relative resistance was defined as the ratio between the IC_{50} value of a given cell line and the IC_{50} value of the wild-type. The effect of VER or CsA on drug resistance was studied by exposing cells to different concentrations of cytostatic drugs in the absence or presence of VER and CsA, respectively.

Semiquantitative Determination of MRP mRNA

RNA was purified by the method of Chomczynski and Sacchi [13]. One-microgram samples of DNase-treated total RNA were reverse-transcribed in reverse transcriptase buffer (Boehringer Mannheim), containing random hexadeoxynucleotides (10 μM), deoxynucleotides, RNAguard RNase inhibitor (20 U), and reverse transcriptase (10 U AMV), at 42° for 60 min in a total volume of 20 μL. These cDNA preparations were diluted 10-fold, and 10-μL aliquots were used for RT–PCR amplification with murine MRP and β-actin primers: (Genbank (GB) accession numbers (www.ncbi.nlm.nih.gov/Web/Genbank) and nucleotide positions in brackets): for murine MRP: sense (GB AF022908 (2552–2574)) 5′CTTATCAGGAGCTGCTAGACCGG, antisense (GB AF022908 (2755–2733)) 5′CACTGTGGGAAGACGAGTTGCTG; for murine β-actin: sense (GB M12481 (48–69)) 5′TGTGATGGTGGGAATGGGTCAG, antisense (GB M1248 (561–540)) 5′TTTGATGTCACGCACGATTTCC.

MRP and β-actin were amplified in 50-μL reactions for five different numbers of cycles: 15, 20, 25, 30, and 35. The PCR buffer (Boehringer Mannheim) contained 200 μM of each deoxynucleotide, 250 nM of each PCR primer, and 1 U Taq DNA polymerase. Ten microlitres of the PCR products were run in 2% agarose gels and visualised by ethidium bromide fluorescence. The amounts of PCR fragments were quantified with Pharmacia ImageMaster gel analysis equipment: both MRP and β-actin were in the linear part of the exponential amplification between 25 and 30 cycles. The β-actin curves overlapped, and differences in MRP could thus be used as semiquantitative estimates. The points of the 30 cycles were used for comparative mRNA measurements.

Determination of P-gp

Preparation of membrane fractions and Western blot analysis were performed as previously described [12]. The monoclonal antibody was C219, obtained from Centocor Diagnostics. Reflectance photometry and control experiments were performed as described in the reference [12].

Western Blot of Topo II

Nuclear extracts of cells in exponential growth were prepared, nuclear protein (100 μg) was electrophoresed, and proteins were transferred to nitrocellulose paper and reacted with Topo II antiserum as previously described [14, 15]. The Topo II antiserum was prepared by Danks and Schmidt with an M_r 70,000 fragment of the COOH terminus of human Topo II expressed in *Escherichia coli* as the immunogen [14]. Reflectance photometry was performed for quantification of the immunoreactive bands [12].

Transport Experiments

The method used to determine drug accumulation was essentially that of Skovsgaard [16]. The standard medium used was a phosphate buffer containing NaCl (57.0 mM), KCl (5.0 mM), $MgSO_4$ (1.3 mM), NaH_2PO_4 (9 mM), and Na_2HPO_4 (51 mM) (pH 7.45), to which 5% (v/v) dialysed bovine serum was added. Steady-state accumulation of [^{3}H]VP16 and DNR was determined at 60 min (N = 3–6). Glucose (10 mM) or NaN_3 (10 mM) was added as indi-

cated. The cellular content of DNR was determined by spectrofluometry after extraction of the drained pellet with 0.3 N HCl: 50% ethanol [16, 17]. Cellular content of [^{3}H]VP16 was measured by extraction of [^{3}H]VP16 from the cell pellet with 0.8 mL of 0.5 N KOH at 80° for 1 hr, and 200 μL was transferred to 10 mL scintillation fluid (Insta-Gel-Plus, Packard BioScience Company) before scintillation counting (TRI-CARB 1900 TR liquid scintillation spectrometer). Efflux and initial uptake of DNR were measured as described elsewhere [18, 19].

Volume Measurements

The volumes of EHR2 and EHR2/VP16 were measured with a Coulter counter set up as described by Hoffmann *et al.* [20]. Cells were washed and resuspended in filtered Ringer's solution (Millipore, pore size 0.45 μm) to 4×10^5 cells/mL. A Coulter counter model ZB equipped with a Coulter Channellyzer C1000 was used for the analysis. The tube orifice diameter was 100 μm. Calibration was performed with polystyrene latex beads 13.5 μm in diameter. The cells were incubated at 37° for 30 min in Ringer's solution before the volumes were measured. The medium cell volume of the cell populations was recorded and stored.

Determination of ATPase Activity

The membrane-associated ATPase activity was determined by colourimetric monitoring of the release of inorganic phosphate from ATP [21, 22]. Membrane proteins were diluted to a protein concentration of 20 μg/mL in ATPase assay medium (ATP 3 mM, KCl 50 mM, $MgSO_4$ 2.5 mM, Tris–HCl 25 mM, dithiothreitol 3 mM, EGTA 0.5 mM, ouabain 2 mM, azide 3 mM; pH 7.0). Each series of experiments was performed in a 96-well microtitre plate containing 50-μL reaction volume/well corresponding to 1 μg protein/well. Incubation with the various drugs was started by transferring the plate from ice to 37° for 1 hr. Water-insoluble drugs were dissolved in ethanol or DMSO. The final concentration of solvent constituted a maximum of 1% v/v of total volume and did not affect ATPase activity. The reaction was stopped by addition of a solution consisting of: ammonium molybdate 0.2% (w/v), sulphuric acid 1.3% (v/v), SDS 0.9% (w/v), trichloroacetic acid 2.3% (w/v), and freshly prepared ascorbic acid 1% (w/v). After incubation at room temperature for 30 min, the amount of released phosphate was quantified colourimetrically (620 nm) using a microplate reader (Bio-Tek). Background values and control experiments were obtained with samples incubated in parallel on ice and samples incubated with vanadate 500 μM, respectively. Background values were routinely subtracted from the measurements.

Statistics

Student's *t*-test was used at a significance level of 5%.

TABLE 1. Drug sensitivity of EHR2 and EHR2/VP16 determined by clonogenic assay

	EHR2	EHR2/VP16
Drug	IC_{50} (μM) (mean ± SEM)	
VP16	0.14 ± 0.01	16.00 ± 4.83 (114.3)*
VP16 + VER 15.7 μM	—	7.60 ± 1.00 (2.1)‡
VP16 + CsA 1.0 μM	—	18.00 ± 3.00 (0.9)‡
DNR	0.019 ± 0.001†	0.108 ± 0.016 (5.7)*
DNR + VER 15.7 μM	—	0.047 ± 0.010 (2.3)‡
DNR + CsA 1.0 μM	—	0.033 ± 0.007 (3.3)‡
VCR	0.0027 ± 0.0007	0.0107 ± 0.010 (4.0)*

Number of experiments = 6.

*Relative resistance = ratio between the IC_{50} of EHR2/VP16 and the IC_{50} of EHR2.

†Ref. 18.

‡Ratio of IC_{50} in the absence versus presence of sensitiser.

RESULTS

Cellular Sensitivity

The sensitivity patterns of EHR2 and EHR2/VP16 are given in Table 1. EHR2/VP16 displayed a high resistance to VP16 (114.3-fold), whereas the subline was 5.7- and 4.0-fold-resistant to DNR and VCR, respectively. EHR2/VP16 showed moderate sensitisation to VP16 and DNR on exposure to VER, whereas exposure to CsA increased the cytotoxicity of DNR, but had no effect on that of VP16.

Expression of MRP and P-gp

Compared with EHR2, the resistant subline, EHR2/VP16, showed an increased expression of MRP mRNA (20-fold) (Fig. 1). The expression of P-gp in EHR2/VP16 was found to be 6 ± 3 arbitrary units (mean of 4 determinations), whereas the expression of P-gp in EHR2 was found earlier to be 12 ± 4 arbitrary units, N = 12 [12].

Immunodetection of Topo II

The level of salt-extractable immunoreactive Topo IIα and Topo IIβ in EHR2/VP16 was reduced by 30% and 40%, respectively, relative to that in EHR2 cells (Fig. 2).

Steady-State Accumulation

The steady-state accumulation of [^{3}H]VP16 and DNR in EHR2 and EHR2/VP16, respectively, is shown in Table 2. Compared with the EHR2 cell line, the steady-state accumulation (60 min) of [^{3}H]VP16 was significantly decreased in EHR2/VP16 (64%), whereas that (at 60 min) of DNR was slightly decreased (17%). In both cases, energy depletion completely corrected the accumulation defects.

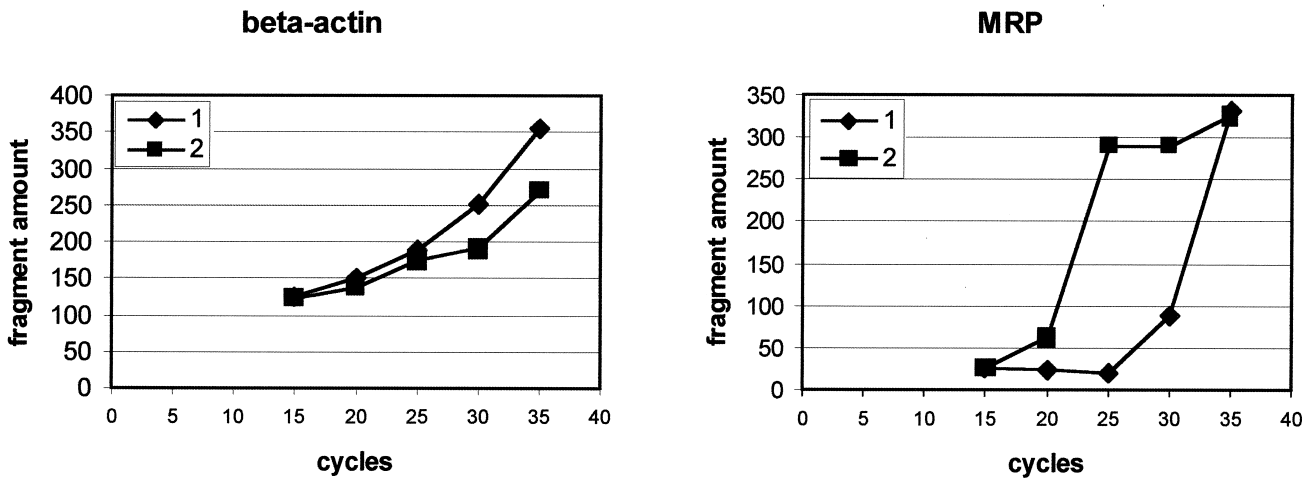

FIG. 1. Semiquantitative RT–PCR for MRP mRNA. β-Actin and MRP were amplified for 15, 20, 25, 30, and 35 cycles. The amounts of PCR fragment in the agarose gels were measured with Pharmacia ImageMaster equipment (arbitrary units). The β-actin curves overlap, which implies that the differences between the MRP curves are quantitative estimates of the MRP mRNA differences. 1: EHR2; 2: EHR2/VP16.

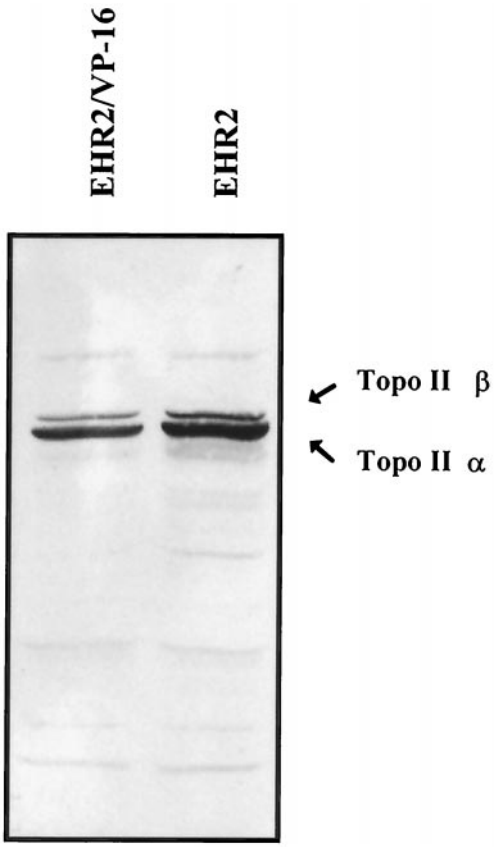

FIG. 2. Western blots of DNA Topo II in nuclear extracts of EHR2 and EHR2/VP16. DNA Topo II was extracted from nuclei with NaCl (1.0 M). Equal amounts of protein (100 μg) were separated, transferred to nitrocellulose paper, and reacted with DNA Topo II antiserum [15]. Reflectance photometry was used for quantification. The area of Topo IIα in EHR2 was 80,568 arbitrary units. The area of the Topo IIα band for EHR2/VP16 was 57,003 arbitrary units. The area of Topo IIβ was 29,402 and 18,410 arbitrary units in EHR2 and EHR2/VP16, respectively.

Figure 3, A and B illustrates the uptake of [^{3}H]VP16 in EHR2 and EHR2/VP16 cells incubated in medium containing sodium azide without glucose. At 30 min, glucose (10 mM) was added to the medium. It appears that glucose induced a pronounced extrusion of [^{3}H]VP16. Addition of VER (25 μM) to the medium had only a moderate effect on drug efflux. Figure 4, A and B shows corresponding experiments in which the uptake of DNR was determined. Addition of VER (25 μM) to the medium decreased the efflux of DNR from EHR2/VP16 to the level of sensitive cells.

Efflux and Initial Uptake of Daunorubicin

The efflux of DNR expressed by $C_t/C_0 \times 100\%$ (C = intracellular drug concentration and t = time) as a function of time is shown in Fig. 5. Compared with EHR2, EHR2/VP16 had a significantly increased efflux. Addition of VER to the efflux medium decreased the DNR efflux from EHR2/VP16 to the level of sensitive cells.

The initial uptake (mean of slopes ± SD; N = 6) of DNR in EHR2 and EHR2/VP16 is given in Table 3. In the glucose-enriched medium, the subline EHR2/VP16 had a significantly decreased initial uptake compared with that of EHR2. Neither addition of VER (11.0 μM) nor depletion of energy (both glycolysis and oxidative phosphorylation) had a significant effect on the initial uptake of DNR in EHR2/VP16. In these media, however, the initial uptake of DNR was not significantly different from that in EHR2.

Cell Volume

The median (± SEM) cell volume for EHR2 and EHR2/VP16 was 1679 ± 20 fl and 1678 ± 19 fl, respectively (N = 3). Thus, the cell volumes of the two cell lines were not different.

TABLE 2. Steady-state accumulation of [^{3}H]etoposide and daunorubicin in EHR2 and EHR2/VP16, respectively

Cell line	[^{3}H]VP16 medium with glucose	[^{3}H]VP16 medium with Na^+-azide	DNR medium with glucose	DNR medium with Na^+-azide	DNR medium with VER 11 μM
EHR2	11.90 ± 2.80	—	1773 ± 59	1902 ± 105	—
EHR2/VP16	4.33 ± 0.43	12.19 ± 2.62	1464 ± 56	1975 ± 78	1878 ± 62

The table shows drug accumulation in pmol/10^6 cells (mean ± SD, N = 3–6).

ATPase Activity

Microsomes prepared from EHR2/VP16 cells had an ATPase activity that differed significantly from that of microsomes derived from P-gp-positive cells (Fig. 6). Both the total and in particular the vanadate-sensitive ATPase activity were very low in the EHR2/VP16 microsomes. Thus, the ATPase activity of EHR2/VP16 was even lower than in the wild-type cell line. Although the ATPase activity in microsomes derived from P-gp-positive cells (EHR2/1.3) was stimulated 1.5- to 2-fold by addition of VBL and VER, these drugs had no effect on the ATPase activity of EHR2/VP16 microsomes. High concentrations of DNR were found to stimulate the ATPase activity in EHR2/VP16 slightly (data not shown), whereas they inhibited the ATPase activity in P-gp-positive cells (EHR2/1.3) (data not shown). In addition, vanadate inhibition of the ATPase activity in EHR2/VP16 microsomes (Fig. 7) had an apparent K_i value that was markedly higher (30.0 μM) than the K_i value obtained for the EHR2/1.3 cell line (8.7 μM).

DISCUSSION

In the present study, we sought to examine the different resistance mechanisms and the functional role of MRP in VP16-resistant tumour cells. EHR2/VP16 displayed high resistance to VP16 (114.3-fold) and was 5.7- and 4.0-fold-resistant to DNR and VCR, respectively. A 30–40% reduction in both Topo IIα and β was found. Since Topo IIβ has only been identified recently, most studies have focused either on total Topo II or on Topo IIα. Most epipodophyllotoxin-selected cell lines exhibited modifications in total Topo II or Topo IIα activity and/or amount [23], whereas decreases in the Topo IIβ level or complete disappearance of the protein have been reported in cell lines resistant to doxorubicin-, amsacrine-, and 9-hydroxy ellipticine [23]. We conclude that quantitative alterations in both Topo IIα and β occurred in EHR2/VP16, and that these alterations partly explain the resistance to VP16 and DNR.

Compared with the parent cell line, EHR2/VP16 showed an increased expression of MRP mRNA. The EHR2/VP16 cell line clearly showed a reduced [^{3}H]VP16 accumulation, which was ATP-dependent. Decreased drug accumulation has been reported for MRP-transfected cell lines and several MDR cell lines with increased expression of MRP [8, 9, 24, 25]. The accumulation profiles for EHR2/VP16 in glucose-enriched medium and in medium depleted of ATP were qualitatively similar to those previously obtained for P-gp-positive cells [12].

Compared with the sensitive cell line EHR2, the EHR2/VP16 cell line had significantly increased [^{3}H]VP16 efflux, whereas DNR efflux from EHR2/VP16 cells was only slightly increased. A previous report describing transport studies performed with membrane vesicles prepared from HL60/ADR cells expressing human MRP was only partly consistent with the present study [26]. Although the amino acid sequences of murine and human MRPs are 88%

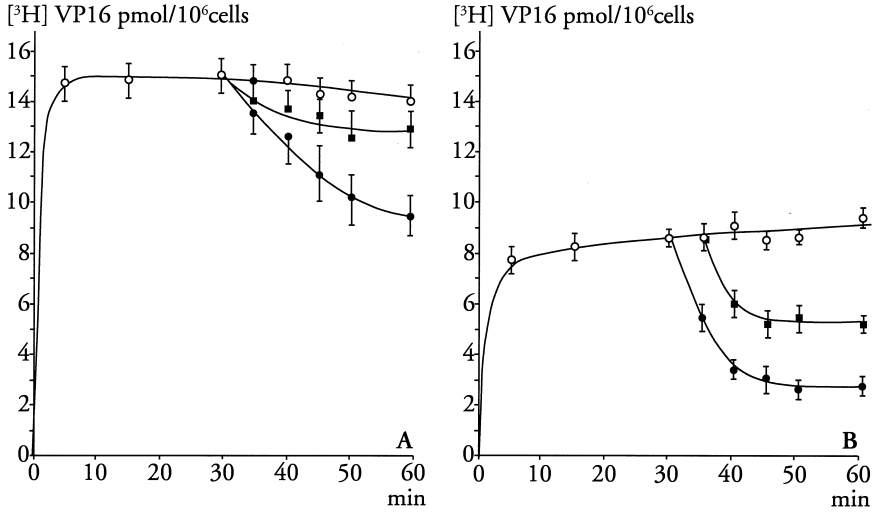

FIG. 3. Accumulation of [^{3}H]VP16 in EHR2 cells (A) and EHR2/VP16 cells (B). The cell suspension was incubated in standard medium without glucose and containing sodium azide (10 mM). At 30 min: ○, NaCl was added to the suspension (control); ●, glucose (10 mM) was added to the suspension; and ■, glucose (10 mM) and verapamil (25 μM) were added to the suspension. Each point represents the mean of 3 determinations and bars represent SEM.

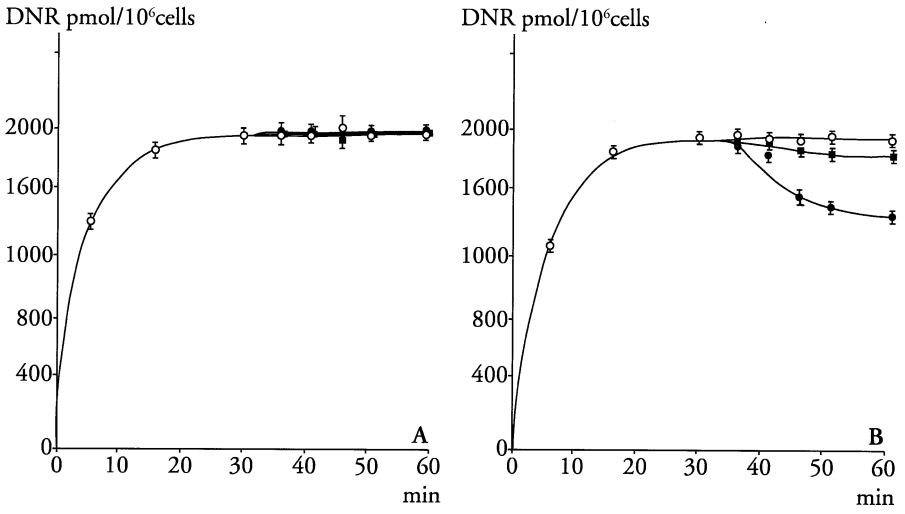

FIG. 4. Accumulation of daunorubicin in EHR2 cells (A) and EHR2/VP16 cells (B). The cell suspension was incubated in standard medium without glucose and containing sodium azide (10 mM). At 30 min: ○, NaCl was added to the suspension (control); ●, glucose (10 mM) was added to the suspension; and ■, glucose (10 mM) and verapamil (25 μM) were added to the suspension. Each point represents the mean of 3 determinations and bars represent SEM.

identical, some biochemical differences were demonstrated. Both proteins were found to transport VP16; however, a significant transport of DNR was detected for human MRP, whereas efflux of DNR was only slightly increased in cells expressing murine MRP. In addition, a considerable variation in the transport properties of MRP-expressing cell lines has been reported [8, 9, 24, 26, 27]. It seems unlikely that methodological differences alone account for the different

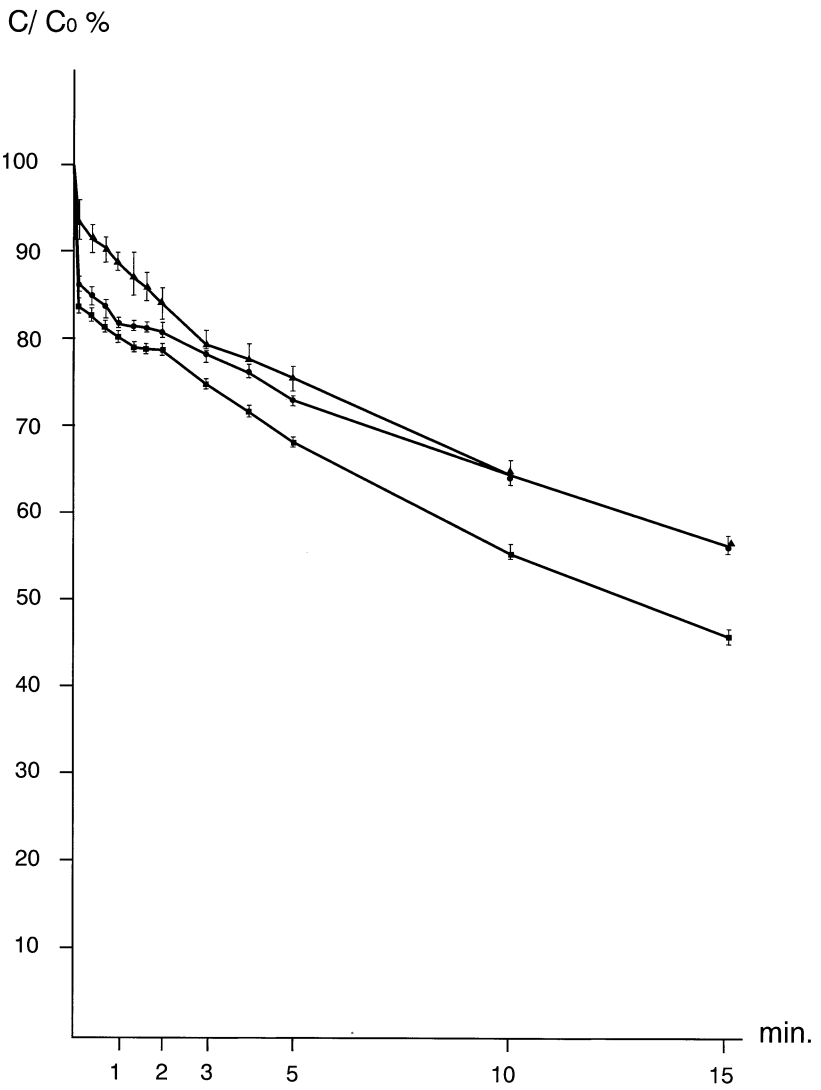

FIG. 5. Efflux of daunorubicin from sensitive (▲) and VP16-resistant Ehrlich ascites tumour cells in drug-free medium with glucose (■) or corresponding medium with verapamil (11 μM) (●). Each point represents the mean of 6 determinations and bars represent SEM.

TABLE 3. Initial uptake of daunorubicin in EHR2 and EHR2/VP16

Cell line	Influx medium with glucose	Influx medium with glucose and verapamil 11 μM	Influx medium with Na^+-azide	Influx medium with Na^+-azide verapamil 11 μM
EHR2*	7.80 ± 1.74	8.32 ± 1.77	8.35 ± 2.19	8.74 ± 2.73
EHR2/VP16	5.78 ± 1.10†	6.05 ± 1.27‡	6.28 ± 1.06‡	6.36 ± 1.09‡

The table shows influx of daunorubicin in pmol/10^6 cells/sec (mean of slopes ± SD, N = 6).
*Ref. 19.
†Significantly different from EHR2 ($P < 0.05$).
‡Not significantly different from either EHR2 or EHR2/VP16.

observations. One possible explanation is that, unlike that of P-gp, drug transport by MRP may be influenced by cellular components that may be cell type-specific. An alternative explanation is that the activities of the proteins encoded by the MRP cDNAs are different. Consistent with this possibility is the fact that the H69R MRP cDNA [2] differs in three amino acid positions when compared to the HL60/ADR MRP cDNA [28], and that single amino acid alterations in P-gp are known to affect transport activity [1]. It is thus possible that genetic polymorphism of the MRP structure may explain the results.

In the present study, addition of VER decreased the efflux of DNR to the level of sensitive cells and had a moderate effect on the VP16 efflux. In contrast, the effect of chemosensitisers in MRP-positive cells has been generally found to be low or moderate [9, 29]. However, our findings were consistent with those of Gaj *et al.* [27], who reported that VER could modulate the MRP-associated accumulation defect (the L-enantiomer was the most potent).

We demonstrated in influx experiments that the initial uptake of DNR in EHR2/VP16 was significantly decreased compared with that in EHR2. Influx has only been measured in two MRP-positive human lung cancer cell lines [30–32]. With respect to influx in a glucose-enriched medium, the results were essentially identical. Contrary to our result, these authors found that ATP depletion increased influx significantly. However, the amount of MRP, and the concomitant decrease in accumulation, might be too small in our cell line to demonstrate such differences clearly.

The current models for MRP function predict that ATP hydrolysis is directly coupled to drug transport [32]. The ability of MRP to bind ATP has been unequivocally demonstrated by photoaffinity labelling of membranes from H69AR cells and MRP-transfected HeLa cells with 8-azido-[^{32}P]ATP [33]. In our study, both the total and particularly the vanadate-sensitive ATPase activity were very low in the EHR2/VP16 microsomes. The ATPase activity of EHR2/VP16 was even lower than in the wild-type cell line. Recently, the ATPase activity of MRP has been determined by Chang *et al.* [34]. They used a multistep procedure to purify human MRP and found that the ATPase activity was moderately stimulated by anthracyclines, vinca alkaloids, and colchicine. They concluded that purified, human MRP

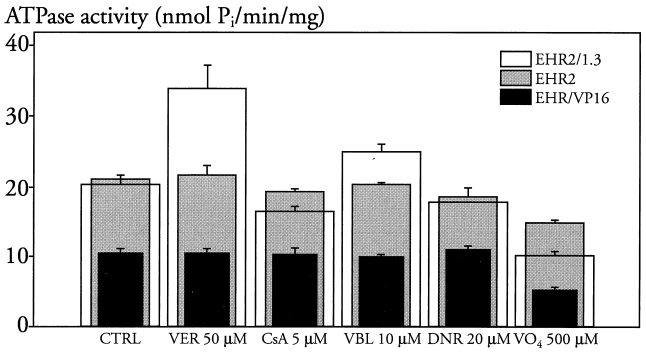

FIG. 6. ATPase activity of microsomes prepared from sensitive (EHR2), P-gp-positive (EHR2/1.3), and MRP-positive (EHR2/VP16) Ehrlich ascites tumour cells. The bars indicate the rate of ATP hydrolysis (nmol/min/mg protein) in the absence of drug (CTRL), with verapamil (50 μM), cyclosporin A (5 μM), vinblastine (10 μM), daunorubicin (20 μM), or vanadate (VO_4) (500 μM) in the incubation medium. Values are means ± SEM (N = 3–6).

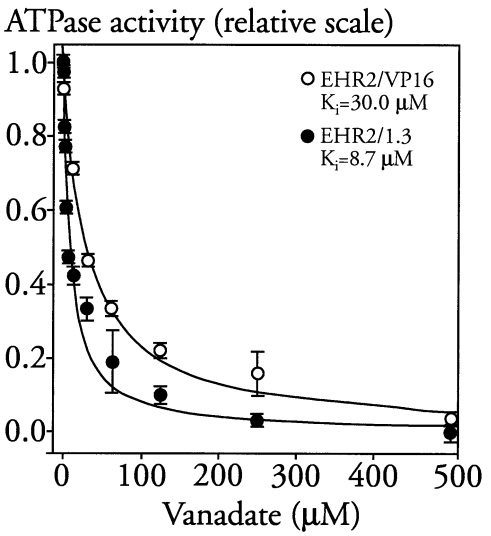

FIG. 7. Vanadate inhibition of the ATPase activity of microsomes prepared from P-gp-positive EHR2/1.3 (●) and MRP-positive EHR2/VP16 (○) cells. The K_i values are 8.7 and 30.0 μM for EHR2/1.3 and EHR2/VP16, respectively ($P < 0.001$). Values are means ± SEM (N = 3).

had an ATPase activity that was at least superficially similar to that of P-gp. The different results could probably be explained by the small amount of MRP in EHR2/VP16. Thus, the MRP activity probably accounts for only a small part of the ATPases in the membrane preparations. The very high K_i value for vanadate inhibition found in the present study could be explained by unspecific inhibition of the background activity of other ATPases. The ATPase activity of human and murine MRP could differ. Lastly, another explanation could be that MRP in some instances may act as a co-transporter. This suggestion corresponds with results reported by Leier *et al.* [35], who demonstrated ATP-dependent transport of glutathione conjugates in MRP-transfected HeLa cells.

In the present study, VER produced only a modest degree of sensitisation to DNR and VP16. In addition, CsA produced moderate sensitisation of the EHR2/VP16 cells to DNR and no sensitisation to VP16. These low sensitisation ratios could be explained by changes in Topo II which were present in EHR2/VP16. It has been a general finding, however, that the effect of chemosensitisers in MRP-positive cells was low or moderate [9, 29].

In conclusion, development of resistance to VP16 in EHR2 is accompanied by a significant reduction in Topo II (α and β) and by increased expression of MRP. The function of MRP was evaluated with VP16 and DNR as substrates. We conclude that MRP displays several points of resemblance to P-gp in its mode of action: 1) like P-gp, MRP causes resistance to a range of hydrophobic drugs; 2) MRP decreases drug accumulation in the cells and this decrease is abolished by omission of energy; 3) MRP increases the efflux of drug from cells. Some major differences, however, exist: compared with P-gp-positive cells, the ATPase activity in MRP-positive cells is found to be low and not able to be stimulated by VER.

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