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Characterisation of Non-P-Glycoprotein Multidrug-Resistant Ehrlich Ascites Tumour Cells Selected for Resistance to Mitoxantrone

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

*Department of Oncology and ‡Department of Haematology, 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 *in vivo* for resistance to mitoxantrone (MITOX). The resistant cell line (EHR2/MITOX) was 6123-, 33-, and 30-fold-resistant to mitoxantrone, daunorubicin, and etoposide, respectively, but retained sensitivity to vincristine. The resistant cells showed moderate sensitisation to mitoxantrone on treatment with verapamil or cyclosporin A. Compared with EHR2, the multidrug resistance-associated protein mRNA was increased 13-fold in EHR2/MITOX. Western blot analysis showed an unchanged, weak expression of P-glycoprotein. Topoisomerase IIα was reduced to one-third in EHR2/MITOX relative to EHR2 cells, whereas topoisomerase IIβ was present in EHR2 but could not be detected in EHR2/MITOX. In the resistant subline, net accumulation of MITOX (120 min) and daunorubicin (60 min) was reduced by 43% and 27%, respectively, as compared with EHR2. The efflux of daunorubicin from preloaded EHR2/MITOX cells was significantly increased. EHR2/MITOX microsomes had a significant basal unstimulated ATPase activity. The apparent K_i value for vanadate inhibition of the ATPase activity in EHR2/MITOX microsomes was not significantly different from the K_i value for P-glycoprotein-positive cells. However, whereas verapamil (50 μM) inhibited the ATPase activity of EHR2/MITOX microsomes, it stimulated the ATPase activity of microsomes derived from P-glycoprotein-positive cells. In conclusion, the resistance in EHR2/MITOX was multifactorial and appeared to be associated with: 1) a quantitative reduction in topoisomerase IIα and β protein; 2) reduced drug accumulation, probably as a result of increased expression of a novel transport protein with ATPase activity; and 3) increased expression of MRP mRNA. BIOCHEM PHARMACOL **60**;3:363–370, 2000. © 2000 Elsevier Science Inc.

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

MITOX¶ is a derivative of dihydroxyanthracenedione. The anthracenedione class of synthetic compounds lacks the amino sugar moiety and the tetracyclic A ring of doxorubicin and other anthracyclines, but contains the planar polycyclic aromatic ring structure. Clinical experience with MITOX has demonstrated its efficacy in the treatment of advanced breast cancer, non-Hodgkin's lymphoma, acute non-lymphoblastic leukaemia, and chronic myelogenous leukaemia [1]. The precise mechanism of action of MITOX remains to be defined, but evidence indicates at least three modes of action: aggregation and compaction of DNA via electrostatic cross-linking, formation of free oxygen radical, and stabilisation of the Topo II–DNA cleavable complex [1]. Topo II alters the topology of DNA by passing an intact double strand of DNA through a transient double-strand break. An intermediate DNA–enzyme complex (the cleavable complex) is formed during this process. By stabilising this complex, Topo II-interacting drugs inhibit the rejoining action of the enzyme, which results in DNA double-strand breaks [2].

The limited data available suggest that resistance to MITOX in tumour cells is multifactorial [1]. Several resistance mechanisms have been described in drug-selected cell lines: 1) The P-gp-mediated MDR phenotype. This is due to the presence of P-gp in the plasma membrane, a "pump" that can extrude a wide range of anticancer drugs [3]; 2) Increased expression of the newly characterised transport protein, MRP, which is also able to extrude anticancer drugs [4]; 3) Increased expression of LRP, recently identi-

‡ 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; LRP, lung resistance-related protein; MDR, multidrug resistance; MITOX, mitoxantrone; 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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fied as human major vault protein [5]; 4) Changes in the intracellular distribution of drug [3]; and 5) Alterations in Topo II [6].

In the present study, we developed a MITOX-resistant cell line *in vivo* in order to elucidate the resistance mechanisms induced by MITOX and the pharmacokinetic properties of a MITOX-resistant cell line.

MATERIALS AND METHODS

Drugs and Chemicals

MITOX was purchased from Lederle. Other drugs and chemicals were obtained as described elsewhere [7].

Tumour Cell Lines and Drug Sensitivity

The tumour cells investigated were the wild-type EHR2 and its corresponding MITOX-resistant subline (EHR2/MITOX). The resistant subline (EHR2/MITOX) was developed *in vivo* in mice by intraperitoneal treatment with increasing doses of MITOX and maintained by treatment with MITOX 2.5 mg/kg × 5 weekly, corresponding to LD10. The cell lines were frozen and all experiments performed within a few passages to avoid experimental variation, owing to evolution of the tumour cells. Drug sensitivity was assessed by clonogenic assay, as previously described by Roed *et al.* [8].

Determination of P-gp and LRP

Preparation of membrane fractions and Western blot analysis were performed as described elsewhere [9]. The monoclonal antibody, C219 (Centocor Diagnostics), was used for detection of P-gp. Immunocytochemical analysis was performed to determine the expression of LRP. The APAAP (immune complexes of alkaline phosphatase and monoclonal anti-alkaline complexes) technique described by Cordell *et al.* [10] was used. The primary monoclonal antibody used was LRP-56 [11]. This antibody was generously provided by Dr Rik J. Scheper, Free University Hospital, Amsterdam, The Netherlands.

Determination of MRP mRNA and mdr1a *and* b *mRNA*

Total RNA was purified by the method of Chomczynski and Sacchi [12]. DNase-treated total RNA (1 μg) was reverse-transcribed with random hexadeoxynucleotides (10 μM), deoxynucleotides, RNAguard RNase inhibitor (20 U), and reverse transcriptase (10 U avian myeloblastosis virus) [7]. These cDNA preparations were diluted and used for RT–PCR amplification of MRP, *mdr1*, and β-actin.

The primers were (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 *mdr1a* and *b: mdr1a* sense (GB M33581 (366–388)) 5′GCTTTGCAAGTGTAGGAAACGTC, *mdr1b* sense (GB M14757 (351–373) 5′CAGAAGCCAGTATTCTGCCAAGC, *mdr1a + b* (common downstream primer, 1a: GB M33581 (620–598), 1b: GB M14757 (602–580)) 5′GCACATCAAACCAGCCTATCTCC; for murine β-actin: sense (GB M12481 (48–69)) 5′TGTGATGGTGGGAATGGGTCAG, antisense (GB M1248 (561–540)) 5′TTTGATGTCACGCACGATTTCC. PCR reactions were run as described in the Ref. 7.

Western Blot of Topo II

Nuclear extracts of log-phase cells were prepared according to the method previously described [13, 14]. Nuclear protein (100 μg) was loaded onto a 5–15% SDS–PAGE gradient gel and electrophoresed overnight. Proteins were transferred to nitrocellulose paper with a Poly Blot apparatus (American Bionetics) and reacted with DNA Topo II antiserum. 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 [13]. The immunoreactive bands were visualised with 5-bromo-4-chloro-3-idolyl phosphate and nitroblue tetrazolium chloride substrates. Reflectance photometry was used to quantify the bands developed on the membranes.

Steady-State Accumulation, Efflux, and Influx

The method used to determine the net accumulation was that of Skovsgaard [15]. The intracellular concentration of MITOX was assessed by spectrophotometry (611 nm). Steady-state accumulation of DNR (5 μM) and MITOX (10 μM) was determined at 60 and 120 min, respectively. Efflux and influx experiments were carried out as previously described [16, 17].

Subcellular Distribution of Daunorubicin

A single cell suspension of 1.0×10^6 cells/mL was incubated at 37° for 1 hr in culture medium (RPMI) containing DNR (2.0 μM). Cells were washed twice in ice-cold PBS before flow cytometry. Cell nuclei were isolated using the CycleTest™ plus DNA reagent from Becton Dickinson Immunocytometry Systems. Mock-treated control cells were analysed in parallel. The binding of DNR to cells and isolated nuclei, respectively, was measured on a FACScan (fluorescence-activated cell scanner, Becton Dickinson Immunocytometry Systems), with excitation by an argon ion laser at 488 nm and measurement of the emitted light at 575 nm. The intensity of the fluorescence is given as the median channel value on a linear scale. For flow cytometry, a cell gate was made in a dot-plot forward scatter versus side scatter mode with linear amplification. The nuclei were

TABLE 1. Drug sensitivity of EHR2 and EHR2/MITOX

Drug	EHR2	EHR2/MITOX
	IC_{50} (μM) (mean ± SEM)	
MITOX	0.0043 ± 0.0003	26.33 ± 2.00 (6123)*
MITOX + VER 15.7 μM	—	4.83 ± 1.03 (5.5)†
MITOX + CsA 1.0 μM	—	2.27 ± 0.67 (11.6)†
DNR	0.019 ± 0.001‡	0.620 ± 0.086 (32.6)*
DNR + VER 15.7 μM	—	0.077 ± 0.010 (8.1)†
DNR + CsA 1.0 μM	—	0.433 ± 0.100 (1.4)†
VP16	0.14 ± 0.01	4.17 ± 0.33 (29.8)*
VCR	0.0027 ± 0.0007	0.0033 ± 0.0010 (1.2)*

Number of experiments = 6.
*Relative resistance = ratio between the IC_{50} of EHR2/MITOX and IC_{50} of EHR2.
†Ratio of IC_{50} in the absence versus presence of sensitiser.
‡Ref. 16.

gated in a similar way, with log amplification for both forward scatter and side scatter.

Determination of ATPase Activity

The MRP-associated ATPase activity was determined according to Borgnia *et al.* [18]. The release of inorganic phosphate from ATP was quantified by a colourimetric method adapted from Chifflet *et al.* [19].

Statistics

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

RESULTS

Cellular Sensitivity

The sensitivity patterns of EHR2 and EHR2/MITOX are shown in Table 1. EHR2/MITOX displayed very high resistance to MITOX (6123-fold) and moderate resistance to DNR and VP16 (33- and 30-fold, respectively), whereas the subline was not cross-resistant to VCR (1.2-fold resistant). EHR2/MITOX showed moderate sensitisation to MITOX on exposure to VER and CsA. The sensitisation ratios (ratio of IC_{50} in the absence versus presence of sensitiser) were 5.5 and 11.6 for VER and CsA, respectively. None of the sensitisers, however, completely reversed resistance in EHR2/MITOX.

Expression of MRP mRNA, P-gp, and LRP

Compared with EHR2, the resistant subline EHR2/MITOX showed a 13-fold increment in MRP mRNA. The expression of P-gp in EHR2/MITOX was 7 ± 4 arbitrary units (mean of 4 determinations). Membrane preparations of EHR2 and EHR2/DNR + were used as controls [9]. The expression of P-gp in EHR2 was determined previously (12 ± 4 arbitrary units, N = 12) [9]. Thus, compared with EHR2, P-gp was unchanged in EHR2/MITOX. This result was confirmed by RT–PCR, as the amount of both *mdr1a* and *b* in EHR2/MITOX was reduced to about fifty percent, as compared with EHR2. Immunocytochemical analysis with the monoclonal antibody, LRP-56, did not show expression of LRP in either EHR2 or EHR2/MITOX (data not shown).

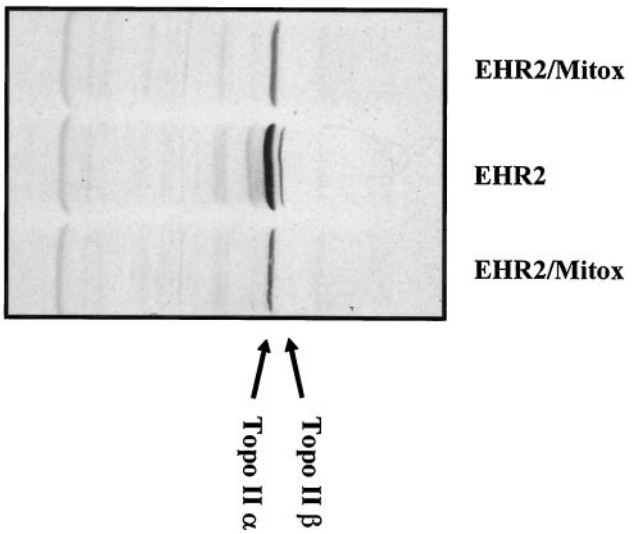

FIG. 1. Western blots of DNA Topo II in nuclear extracts of EHR2 and EHR2/MITOX. DNA Topo II was extracted from nuclei with NaCl (1.0 M). Equal amounts of protein (100 μg) were separated on a 5–15% SDS–PAGE gel, transferred to nitrocellulose paper, and reacted with DNA Topo II antiserum [12]. Reflectance photometry was used for quantification. The area of Topo IIα in EHR2 was 102,113 arbitrary units. The areas of the Topo IIα bands for EHR2/MITOX were 32,584 and 30,102 arbitrary units, respectively. The area of Topo IIβ in EHR2 was 17,572 units, whereas Topo IIβ could not be detected in EHR2/MITOX.

Immunodetection of Topo II

The amount of salt-extractable immunoreactive Topo IIα in EHR2/MITOX was reduced to one-third relative to that in EHR2 cells, whereas Topo IIβ could not be detected in EHR2/MITOX (Fig. 1).

Steady-State Accumulation, Efflux, and Initial Uptake

Net accumulation of MITOX and DNR in EHR2 and EHR2/MITOX, respectively, is shown in Table 2. Compared with the EHR2 cell line, net accumulation (120 min) of MITOX was significantly decreased in EHR2/MITOX ($P < 0.001$). Depletion of energy increased the net accumulation of MITOX both in EHR2/MITOX and in sensitive EHR2 cells. In medium including Na^+-azide,

TABLE 2. Net accumulation of mitoxantrone and daunorubicin in EHR2 and EHR2/MITOX, respectively

Cell line	MITOX glucose-enriched medium	MITOX medium including Na^+-azide	DNR glucose-enriched medium	DNR medium including Na^+-azide	DNR medium including VER 11 μM
EHR2	2193 ± 139	3857 ± 409	1773 ± 59	1902 ± 115	—
EHR2/MITOX	1256 ± 122	3149 ± 402	1293 ± 86	1881 ± 86	1474 ± 167

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

accumulation in the two types of cells was not significantly different. Compared to the wild-type cell line, the steady-state accumulation (60 min) of DNR was significantly decreased in EHR2/MITOX ($P < 0.001$). Application of VER increased the accumulation, but did not correct the accumulation defect in EHR2/MITOX ($P < 0.001$), whereas energy depletion completely corrected the defect.

Efflux (washout kinetics) of daunorubicin is shown in Fig. 2. Data are expressed by $C_t/C_0 \times 100\%$ as a function of time. Efflux of DNR from EHR2/MITOX was signifi-

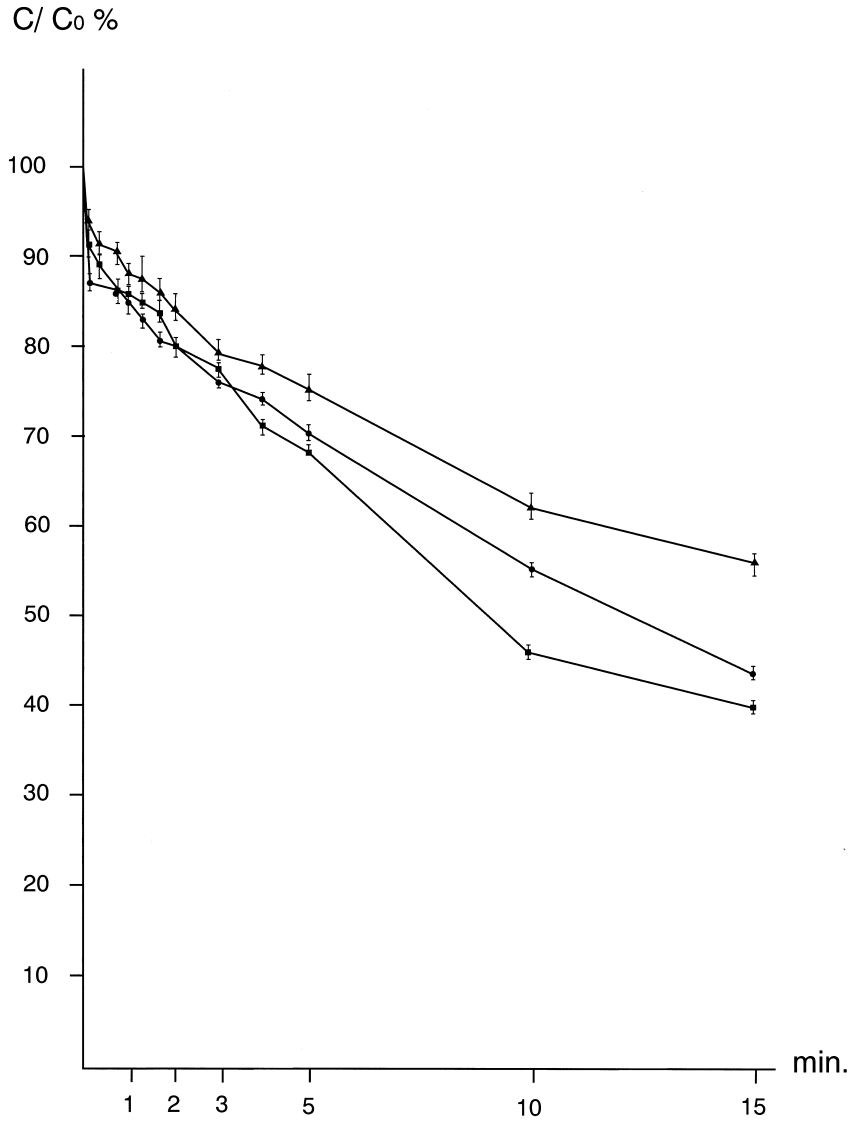

FIG. 2. Efflux of daunorubicin from wild-type (▲) and MITOX-resistant EHR2 cells in drug-free medium with glucose (■) or a corresponding medium with verapamil (11.0 μM) (●). Each point represents the mean of 6 determinations and bars represent SEM values.

TABLE 3. Initial accumulation of daunorubicin in EHR2 and EHR2/MITOX

Cell line	Influx glucose-enriched medium	Influx glucose-enriched medium verapamil 11 μM	Influx medium including Na^+-azide	Influx medium including Na^+-azide verapamil 11 μM
EHR2*	7.80 ± 1.74	8.32 ± 1.77	8.35 ± 2.19	8.74 ± 2.73
EHR2/MITOX	4.87 ± 0.57†	5.11 ± 0.90‡	5.69 ± 0.67‡	5.69 ± 0.71‡

The table shows influx of daunorubicin in pmol/10^6 cells/sec (mean of slopes ± SD, N = 6).
†Significantly different from EHR2 ($P < 0.01$).
‡Significantly different from EHR2 ($P < 0.05$), not significantly different from EHR2/MITOX.
*Ref. 17.

cantly increased. Addition of VER did not affect the efflux of DNR from EHR2/MITOX significantly ($P > 0.05$).

The initial uptake (mean of slopes ± SD; $N = 6$) of DNR in EHR2 and EHR2/MITOX is shown in Table 3. In all experiments, EHR2/MITOX had significantly decreased initial uptake compared to EHR2. Neither addition of VER (11.0 μM) nor depletion of energy (both glycolysis and oxidative phosphorylation) significantly affected the initial uptake of DNR in EHR2/MITOX.

Subcellular Distribution of Daunorubicin

The nuclear and total cellular fluorescence of EHR2 and EHR2/MITOX after a 1-hr exposure to DNR (2.0 μM) are given in Table 4. The nuclear/total cellular DNR fluorescence ratio was 0.43 for both cell types, which suggests that the subcellular distribution was similar in EHR2 and EHR2/MITOX. The result was confirmed by confocal microscopy (data not shown).

ATPase Activity

The ATPase activity of microsomes prepared from EHR2/MITOX, EHR2, and a P-gp-positive subline EHR2/1.3, respectively, is shown in Fig. 3. The basal unstimulated ATPase activity of EHR2/MITOX was 9.6 ± 1.0 nmol P_i/min/mg, whereas the unstimulated ATPase activity of EHR2/1.3 was 21.0 ± 0.7 nmol P_i/min/mg. Vanadate inhibition of the ATPase activity in EHR2/MITOX microsomes had an apparent K_i value (6.9 μM) that was not significantly different from the K_i value obtained for an MDR cell line previously shown to overexpress P-gp (8.7 μM) (Fig. 4). Although the ATPase activity of microsomes derived from P-gp-positive cells was greatly stimulated by the addition of VER (50 μM), this concentration of the drug inhibited the ATPase activity of EHR2/MITOX microsomes. High concentrations of DNR were found to stimulate the ATPase activity in EHR2/MITOX, whereas VBL and CsA inhibited the ATPase activity.

TABLE 4. Subcellular distribution of daunorubicin

Cell line	Total cellular fluorescence of daunorubicin (mean ± SEM)	Total nuclear fluorescence of daunorubicin (mean ± SEM)
EHR2	437 ± 3 (N = 32)*	190 ± 4 (N = 22)†
EHR2/MITOX	387 ± 6 (N = 33)*	166 ± 6 (N = 22)†

* Four independent experiments.
† Three independent experiments.

DISCUSSION

The available data suggest that most MITOX-resistant tumour cell lines have an atypical resistance profile, displaying only partial cross-resistance to other compounds in the MDR family. Increased expression of P-gp has been observed [20], but most MITOX-resistant sublines did not overexpress this protein [21–24]. In accordance with these results, our EHR2/MITOX cell line did not overexpress P-gp as compared with EHR2. EHR2/MITOX showed a significantly increased expression of MRP. Conversely, Futscher *et al.* [25] found that none of four independently MITOX-selected cell lines exhibited an increased expression of MRP.

LRP has recently been detected in two MITOX-resistant cell lines [25]. In the present study, immunocytochemical analysis with the monoclonal antibody LRP-56 did not show expression of LRP in EHR2/MITOX. LRP-56 was developed by immunisation of mice with 2R120 cells (P-gp-negative human non-small cell lung cancer). Although sequence analysis has indicated an 87.7% amino acid identity between LRP and the major vault protein from *Rattus norvegicus* [26], it is possible that the LRP-56 antibody did not recognise murine major vault protein. Hence, the presence of LRP in EHR2/MITOX cannot be ruled out.

Our EHR2/MITOX subline clearly showed ATP-dependent, reduced accumulation of MITOX and DNR. Compared with the wild-type cell line (EHR2), the EHR2/MITOX cell line had a significantly increased efflux of DNR. In another study [7], we investigated the kinetics of DNR transport in a VP16-resistant EHR2 subline (EHR2/VP16). This subline had approximately the same content of MRP as EHR2/MITOX. A comparison of EHR2/MITOX with EHR2/VP16 and other MRP-positive cell lines [27] showed several similarities in the transportation of DNR. However, VER had only a limited effect on the accumulation of DNR in EHR2/MITOX, and the drug had no effect on the efflux of DNR from EHR2/MITOX. In contrast,

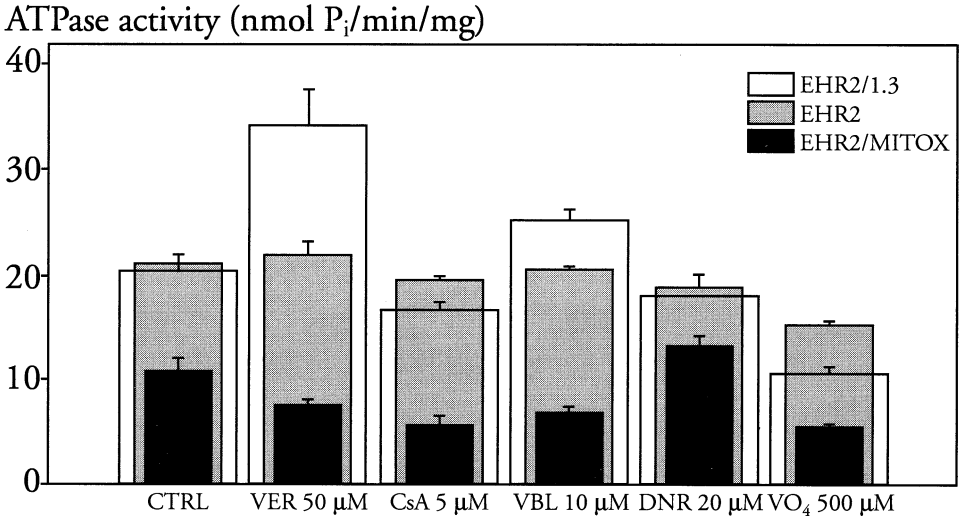

FIG. 3. ATPase activity of microsomes prepared from wild-type EHR2, P-gp-positive EHR2/1.3, and EHR2/MITOX Ehrlich ascites tumour cells. The bars indicate the rate of ATP hydrolysis (nmol/min/mg protein) without drug (CTRL), and with vinblastine (10 μM), daunorubicin (20 μM), verapamil (50 μM), cyclosporin A (5 μM), or vanadate (VO_4) (500 μM) in the incubation medium. Values are means ± SEM (N = 3–6).

VER was able to increase accumulation and decrease efflux of DNR in EHR2/VP16 to the level of sensitive cells [7].

EHR2/MITOX microsomes had a significant basal unstimulated ATPase activity. Chang *et al.* [28], who used highly purified human MRP, found that the protein possessed significant basal unstimulated ATPase activity, which was stimulated by doxorubicin, VCR, VBL, and colchicine, but not by DNR. Thus, the expression of MRP could explain the basal activity found in EHR2/MITOX microsomes. However, in the present study, the ATPase activity was stimulated by DNR, but not by VBL. Moreover, the basal and drug-inducible ATPase activity of EHR2/MITOX microsomes was significantly different from the ATPase activity of MRP-positive EHR2/VP16 microsomes [7]. In the two studies, the parent cell line, the preparation of microsomes, and the methods for investigation of ATPase activity were identical. Furthermore, approximately the same amount of MRP was found in the two sublines. This strongly suggests that MRP did not account for the ATPase activity seen in EHR2/MITOX. Taken together, it is unlikely that expression of MRP is a major determinant for either transport of cytostatics, or resistance, or the ATPase activity in EHR2/MITOX.

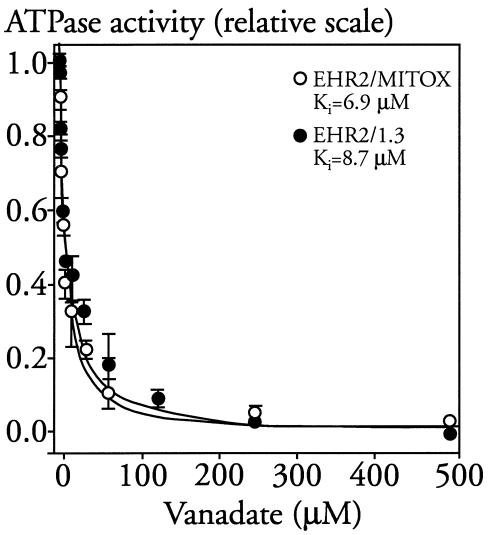

FIG. 4. Vanadate inhibition of the ATPase activity of microsomes prepared from P-gp-positive EHR2/1.3 (●) and EHR2/MITOX (○) cells. The K_i values are 8.7 and 6.9 μM for EHR2/1.3 and EHR2/MITOX, respectively. Values are means ± SEM (N = 3–6).

The ATPase activity of EHR2/MITOX microsomes was similar to that of P-gp-positive microsomes in several aspects. The drug-inducible activity, however, showed some major differences. A substantial activation of P-gp ATPase activity by VER has consistently been reported [29]. In the present study, VER (50 μM) inhibited the ATPase activity of EHR2/MITOX microsomes. Furthermore, high concentrations of DNR were found to stimulate the ATPase activity in EHR2/MITOX. There are two possible explanations: One is the presence of mutant P-gp. Several mutations have been shown to affect basal and drug-stimulated ATPase activity of purified, reconstituted human P-gp [30]. Mutations could change P-gp, thus making the epitope unrecognisable by the primary antibody (C219). However, the finding of decreased *mdr1a* and *mdr1b* RNA in EHR2/MITOX makes the presence of mutated P-gp in this cell line unlikely.

A second explanation is the presence of another transport protein. As mentioned, various groups have identified cell lines selected in MITOX that display phenotypes comparable to EHR2/MITOX [20–24]. Lee *et al.* [31] recently described a human MCF-7 cell line (MCF-7/AdVp) selected in a combination of doxorubicin and VER with an identical phenotype. In several of these cell lines,

the drug kinetics were similar to that of EHR2/MITOX [21–24, 31]. Neither P-gp nor MRP was increased in the above-mentioned cell lines [25, 31, 32]. Amplification of a new ATP-binding transporter (*MXR1*, *2*), was recently demonstrated in MCF-7/AdVp [33]. Furthermore, Lage and Dietel [34] have cloned and characterised a glypican-encoding gene, *MXR7*, which was expressed in a MITOX-resistant EPG85-257RNOV subline. *MXR7* seems to be identical to a glypican gene, *GCP3* [35], and has shown a high degree of similarity to a rat intestinal development protein designated OCI-5. It is possible that one of these genes could be expressed in EHR2/MITOX. Investigations are currently in progress to clone the murine homologues to the human genes implicated in MITOX resistance.

Redistribution of drug away from target sites as opposed to an overall decrease in the total cellular accumulation of drug may be a significant factor in determining resistance. Dietel and Seidel [36] reported an intensive formation of surface vesicles associated with the emergence of resistance to MITOX in gastric carcinoma cells. In the present study, the nuclear/total cellular ratio of the DNR fluorescence ratio was, however, similar in EHR2/MITOX and EHR2 cells. This result is consistent with the findings of Lee *et al.* [31]. It is thus unlikely that DNR resistance in EHR2/MITOX is caused by changes in the subcellular distribution of drug. This finding, however, does not rule out that the marked resistance to MITOX could be explained by drug being trapped in the Golgi complex [37].

Two Topo II isoforms, Topo IIα (170 kDa) and Topo IIβ (180 kDa), exist in animal cells. Of these, Topo IIα is the more sensitive to TOPO II-inhibiting drugs [38]. The relative amount of each isoenzyme may be a determinant of resistance to anticancer drugs, and the role of each isoform in resistance may differ [6]. In the present study, we found that Topo IIβ completely disappeared in EHR2/MITOX, whereas Topo IIα in EHR2/MITOX was reduced to about one-third relative to that in EHR2 cells. One explanation could be that MITOX preferentially produces cytotoxic damage via Topo IIβ rather than Topo IIα. Accordingly, Harker *et al.* [20] demonstrated a marked reduction in, if not absence of, immunodetectable Topo IIβ protein in a MITOX-resistant leukaemia cell line. However, decreases in the Topo IIβ level or the complete disappearance of the protein has also been reported in doxorubicin-resistant cell lines [39]. We conclude that quantitative alterations in both Topo IIα and β occur in EHR2/MITOX cells and that these alterations contribute to its resistant phenotype.

Taken together, these results indicate that resistance to MITOX in an EHR2 subline is multifactorial. The resistance appeared to be associated with: 1) a quantitative reduction in Topo IIα and β protein; 2) reduced drug accumulation, probably as a result of increased expression of a novel transport protein with ATPase activity; and 3) increased expression of MRP.

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