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ORIGINAL ARTICLE

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Influence of chemosensitizers on resistance mechanisms in daunorubicin-resistant Ehrlich ascites tumour cells

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Abstract *Aim:* To determine whether treatment with chemosensitizers influences the development of the drug-resistant phenotype. *Methods:* Three sublines were developed from the sensitive Ehrlich ascites tumour cell line (EHR2) and six sublines from the EHR2/DNR cell line positive for P-glycoprotein (PGP) by treatment with daunorubicin (DNR), a combination of DNR and verapamil (VER), or a combination of DNR and cyclosporin A (CsA). A clonogenic assay was used to determine resistance, the expression of PGP, the multidrug resistance associated protein (*Mrp1*) and topoisomerase IIα and β were measured by Western blotting, and reverse transcriptase-polymerase chain reaction was used for determination of *mdr1a* and *b*, and *Mrp1* mRNA. *Results:* Compared with the EHR2 cell line, the amounts of *mdr1a* mRNA increased significantly in all sublines except EHR2/DNR, whereas *mdr1b* mRNA levels were unchanged. Compared with the EHR2 subline selected in DNR alone, the levels of *mdr1a* mRNA and PGP were significantly lower in the EHR2 sublines selected in the presence of chemosensitizer. Furthermore, *mdr1a* mRNA and PGP were unchanged in all cotreated sublines selected from the PGP-positive EHR2/DNR cell line. The mRNA and protein levels of *Mrp1* did not change significantly in any of the cell lines. Only one DNR plus VER-selected subline showed a decrease in topoisomerase IIα (one-third as compared with EHR2). All DNR plus CsA-selected sublines showed significantly less resistance than the corresponding DNR- and DNR plus VER-selected sublines. The effect of VER and CsA on cytotoxicity was retained in all cell lines treated with chemosensitizer. *Conclusions:* Selection in chemosensitizer resulted in a decrease in the expression of *mdr1a* and PGP. These chemosensitizers do not seem to influence *Mrp1* expression or topoisomerase II. Selection in CsA may retard the development of resistance.

Keywords Cell line · Cyclosporin A · Daunorubicin · Resistance · Verapamil

Abbreviations *CsA* cyclosporin A · *DNR* daunorubicin · *MDR* multidrug resistance · Mrp1 murine multidrug resistance-associated protein · *PGP* P-glycoprotein · *RT-PCR* reverse transcriptase-polymerase chain reaction · *VER* verapamil

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Introduction

Drug resistance is one of the major obstacles to effective cancer chemotherapy. Despite the multitude of chemotherapeutic agents available, tumour cells frequently become refractory to drug treatment. Understanding the mechanisms underlying the development of drug-resistant tumours is a prerequisite for devising rational therapeutic approaches to circumvent or avoid the emergence of such refractory tumours.

Two main categories of resistance mechanisms have been described in anthracycline-selected cell lines. The first category concerns mechanisms resulting in a reduced intracellular concentration of drug. This multidrug resistance (MDR) phenotype is conferred by at least two proteins: P-glycoprotein (PGP), encoded by the *mdr1* gene [16, 17], and a more recently identified protein, multidrug resistance-associated protein (*Mrp1*) [15]. Both PGP and *Mrp1* belong to the ATP-binding cassette superfamily of transport proteins. The proteins serve as energy-dependent drug efflux pumps and the reduced intracellular concentration of drug observed in resistant cells is normally attributed to increased efflux

[15, 16, 17]. The second category of resistance involves alterations affecting the drug target, the enzyme topoisomerase II (TOPO II) [3]. DNA TOPO II is involved in several aspects of DNA metabolism, in particular genetic recombination, DNA transcription, and chromosome segregation.

A considerable body of experimental work shows that certain classes of membrane-active drugs are capable of circumventing PGP-mediated resistance to various degrees (reviewed in [10, 17]). The compounds that modulate PGP-mediated resistance in vitro represent a wide range of chemical structures, including calcium channel blockers, calmodulin inhibitors, steroids, and immunosuppressive agents. Clinical trials combining a modulator with chemotherapeutic agents are in progress [6, 9]. Studies have indicated that the expression of PGP may be modulated by chemosensitizers [2, 14]. In order to evaluate the influence of chemosensitizers on the evolution of the drug-resistant phenotype, we treated a wild-type Ehrlich ascites tumour cell line and its PGP-positive counterpart with combinations of daunorubicin (DNR) and either verapamil (VER) or cyclosporin A (CsA). The results of this study indicated that selection in the presence of VER or CsA decreases the expression of *mdr1a* and PGP. These chemosensitizers did not seem to influence *Mrp1* or topoisomerase II levels. Selection in CsA may retard the development of resistance.

Materials and methods

Tumour cell lines

The wild-type Ehrlich ascites tumour (EHR2) was used in the experiments. The sublines were developed and maintained in vivo by intraperitoneal treatment with DNR alone or with a combination of DNR and chemosensitizer. All cell lines were maintained in first-generation hybrids of female NMRI and male inbred DBA/2 mice by weekly transplantation (when possible) of 1.5×10^7 cells per mouse. The treatment protocols are given in Table 1. In the resistant cell lines, the doses of DNR (in milligrams per kilogram four times weekly) used for the development of the sublines (DNR 1.6 mg/kg corresponds to LD_{10}) are indicated by a suffix. The suffix V indicates that the subline was developed in a combination of DNR and VER, and the suffix C indicates that the subline was developed in a combination of DNR and CsA. The DNR-selected subline EHR2/1.6 reached 36 passages at 52 weeks, whereas the coselected sublines EHR2/1.6V and EHR2/1.6C reached 36 passages at 70 and 88 weeks, respectively. Furthermore, cotreatment with VER delayed tumour growth in the sublines EHR2/DNR/0.4V (12 passages were reached after 16 weeks). All other sublines were transplanted weekly. All experiments were performed 6–7 days after transplantation.

Clonogenic assay

The cytotoxicity of DNR was measured by clonogenic assay as described previously [24]. Briefly, a single-cell suspension (5×10^3 cells) with the desired drug concentration was plated in soft agar on a feeder layer containing sheep red blood cells and mercaptoethanol (plating efficiency ≥60%). Continuous drug exposure was used. Colonies were counted after 1 week. Each experiment was performed in triplicate and repeated six times. IC_{50} values were defined as the drug concentrations inhibiting colony formation by 50%. 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 cell line.

The effect of chemosensitizer on drug resistance was studied by exposing cells to different concentrations of DNR in the absence or presence of VER or CsA.

Determination of PGP and *Mrp1*

Preparations of membrane fractions and Western blot analysis were performed as described elsewhere [21]. The monoclonal antibodies used were C219 (Centocor Diagnostics, Philadelphia, Pa.) and anti-MRP (MRPr1) (Catalogue no. 475726; Calbiochem-Novabiochem Corporation, San Diego, Calif.). PGP was visualized using peroxidase-conjugated $F(ab')_2$ fragments of affinity-purified sheep anti-mouse IgG (Medac, Hamburg, Germany). *Mrp1* was visualized by chemiluminescence using a BM chemiluminescence blotting substrate kit from Boehringer Mannheim (Mannheim, Germany). Reflectance photometry and control experiments were performed as described previously [21]. The experiments were repeated three to eight times with 3×10^8 cells from three animals each time.

RNA isolation and cDNA synthesis

Total RNA was purified by the method of Chomczynski and Sacchi [5]. Aliquots of RNA (10 μg) were treated with DNase (Boehringer Mannheim) in the presence of RNAguard RNase inhibitor. Samples containing DNase-treated total RNA (1 μg) were reverse transcribed in reverse transcriptase buffer (Boehringer Mannheim), with random hexadeoxynucleotides (10 μ*M*), deoxynucleotides, RNAguard RNase inhibitor (20 U), and reverse transcriptase (10 U AMV). These cDNA preparations were diluted tenfold, and 10-μl aliquots were used for RT-PCR amplification of mRNA for

Table 1. Treatment protocols for Ehrlich ascites tumour cell lines

Cell line	DNR (mg/kg ×4 weekly)	VER (45 mg/kg ×4 weekly)	CsA (15 mg/kg ×4 weekly)
EHR2	–	–	–
EHR2/1.6	1.6	–	–
EHR2/1.6V	1.6	From passage 1	–
EHR2/1.6C	1.6	–	From passage 1
EHR2/DNR	0.1 (96 passages)	–	–
EHR2/DNR/0.1	0.1 (108 passages)	–	–
EHR2/DNR/0.1 V	0.1 (108 passages)	From passage 97	–
EHR2/DNR/0.1C	0.1(108 passages)	–	From passage 97
EHR2/DNR/0.4	0.1 for 96 passages 0.4 from passage 97	–	–
EHR2/DNR/0.4V	0.1 for 96 passages 0.4 from passage 97	From passage 97	–
EHR2/DNR/0.4C	0.1 for 96 passages 0.4 from passage 97	–	From passage 97

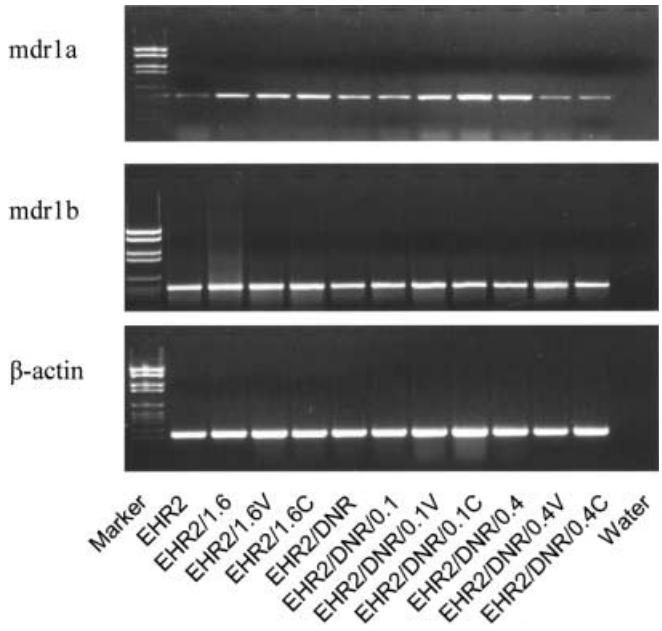

Fig. 1. Qualitative RT-PCR amplification of *mdr1a* and *b* mRNA in the sensitive EHR2 cell line, in cell lines selected in DNR (EHR2/1.6, EHR2/DNR, EHR2/DNR/0.1, EHR2/DNR/0.4), in cell lines selected in DNR plus VER (EHR2/1.6V, EHR2/DNR/0.1V, EHR2/DNR/0.4V), and in cell lines selected in DNR plus CsA (EHR2/1.6C, EHR2/DNR/0.1C, EHR2/DNR/0.4C)

mdr1a, *mdr1b* and *Mrp1*, respectively. Amplification of mRNA for the housekeeping gene β-actin was used as control.

Determination of *mdr1a* and *mdr1b*

The primers used for the PCR amplification of mRNA for *mdr1a* and *b* are listed below together with their GenBank accession numbers (http://www.ncbi.nlm.nih.gov/Web/Genbank) and nucleotide positions in parentheses:

- *mdr1a* sense 5′GCTTTGCAAGTGTAGGAAACGTC (M33581, 366–388)
- *mdr1b* sense 5′CAGAAGCCAGTATTCTGCCAAGC (M14757, 351–373)
- *mdr1a*+*b* common downstream primer 5′GCACATCAAACCAGCCTATCTCC (1a: M33581, 620–598; 1b: M14757, 602–580)

The primers for murine β-actin were:

- sense 5′TGTGATGGTGGGAATGGGTCAG (M12481, 48–69)
- antisense 5′TTTGATGTCACGCACGATTTCC (M1248, 561–540)

PCR reactions (50 µl) were run on a Perkin Elmer 9600 Thermocycler: 35 cycles of 94°C 30 s, 55°C 30 s and 72°C 30 s. The PCR buffer (Boehringer Mannheim) contained 10 m*M* Tris/HCl, pH 9.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 0.01% (v/v) Triton X-100, 200 µ*M* of each deoxynucleotide, 250 n*M* of each PCR primer, and 1 U Taq DNA polymerase. For qualitative determination the PCR products were run in 2% agarose gels and visualized by ethidium bromide fluorescence. In addition, *mdr1a* was semiquantified according to the following procedure. mRNA of *mdr1a* and β-actin were subjected to five amplifications using different numbers of cycles (15, 20, 25, 30, and 35 cycles) each comprising 94°C 30 s, 55°C 30 s and 72°C 30 s. Both *Mdr1a* and β-actin were in the linear part of the exponential amplification between 25 and 30 cycles. The β-actin curves overlapped

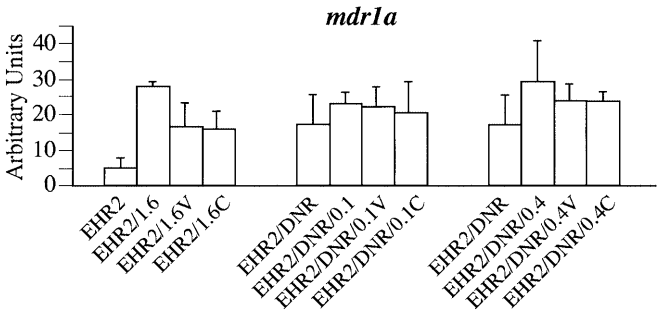

Fig. 2. Expression of *mdr1a* mRNA in the sensitive EHR2 cell line, in cell lines selected in DNR (EHR2/1.6, EHR2/DNR, EHR2/DNR/0.1, EHR2/DNR/0.4), in cell lines selected in DNR plus VER (EHR2/1.6V, EHR2/DNR/0.1V, EHR2/DNR/0.4V), and in cell lines selected in DNR plus CsA (EHR2/1.6C, EHR2/DNR/0.1C, EHR2/DNR/0.4C). *Bars* SD

and therefore differences in *Mdr1a* were used as semiquantitative estimates. The points from the 30-cycle amplifications were used for comparative mRNA measurements. The experiments were repeated three times with 3×10^8 cells from three animals each time.

Semiquantitative determination of *Mrp1* mRNA

The primers used are listed below together with their GenBank accession numbers (http://www.ncbi.nlm.nih.gov/Web/Genbank) and nucleotide positions in parentheses:

- *Mrp1*: sense 5′CTTATCAGGAGCTGCTAGACCGG (AF022908, 2552–2574), antisense 5′CACTGTGGGAAGACGAGTTGCTG (AF022908, 2755–2733)
- murine β-actin: sense 5′TGTGATGGTGGGAATGGGTCAG (M12481, 48–69), antisense 5′TTTGATGTCACGCACGATTTCC (M1248, 561–540)

A semiquantitative determination of *Mrp1* was performed as described for *mdr1a*. The experiments were repeated three times.

Western blot of topoisomerase II

TOPO II was extracted from the nuclei of log-phase cells (from three animals) with 1.0 *M* NaCl, as described elsewhere [7, 12]. To detect TOPO II, 100 µg nuclear protein was separated on a 5–15% SDS-PAGE gradient gel. Proteins were then transferred to nitrocellulose paper with a Poly Blot apparatus (American Bionetics, Hayward, Calif.) and probed with DNA TOPO II antiserum. Danks and Schmidt prepared the TOPO II antiserum with a M_r 70,000 fragment of the COOH terminus of human TOPO II expressed in *Escherichia coli* as the immunogen [7]. The immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride substrates. TOPO IIα and β were semiquantified by reflectance photometry with the sensitive cell line EHR2 as reference.

Steady-state accumulation

The procedures were performed as described by Skovsgaard [25]. Steady-state accumulation of DNR was determined at 60 min. The cellular content of DNR was determined by spectrofluorimetry after extraction of the drained pellet with 0.3 *N* HCl/50% ethanol (experiments were repeated three times with six to eight animals each time).

Statistics

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

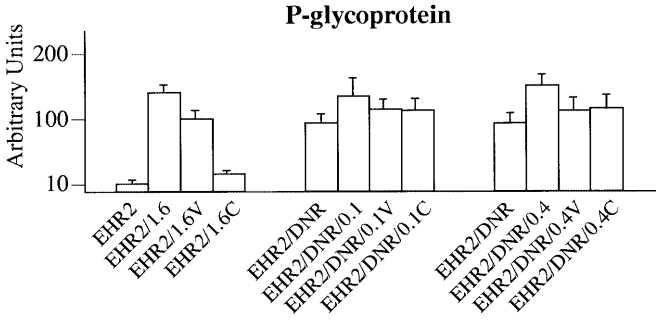

Fig. 3. Expression of PGP in the sensitive EHR2 cell line, in cell lines selected in DNR (EHR2/1.6, EHR2/DNR, EHR2/DNR/0.1, EHR2/DNR/0.4), in cell lines selected in DNR plus VER (EHR2/1.6V, EHR2/DNR/0.1V, EHR2/DNR/0.4V), and in cell lines selected in DNR plus CsA (EHR2/1.6C, EHR2/DNR/0.1C, EHR2/DNR/0.4C). *Bars* SD

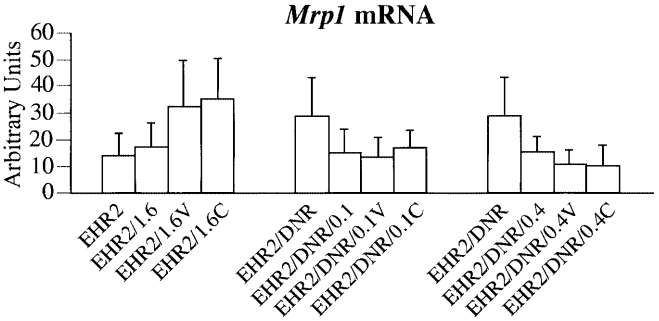

Fig. 4. Expression of *Mrp1* mRNA in the sensitive EHR2 cell line, in cell lines selected in DNR (EHR2/1.6, EHR2/DNR, EHR2/DNR/0.1, EHR2/DNR/0.4), in cell lines selected in DNR plus VER (EHR2/1.6V, EHR2/DNR/0.1V, EHR2/DNR/0.4V), and in cell lines selected in DNR plus CsA (EHR2/1.6C, EHR2/DNR/0.1C, EHR2/DNR/0.4C). *Bars* SD

Results

Expression *mdr1a* and *mdr1b* mRNA and PGP

The wild-type cell line was adapted to grow in DNR and a combination of DNR and chemosensitizers, respectively. Compared with EHR2, the *mdr1a* mRNA levels increased significantly in all these sublines, whereas *mdr1b* mRNA levels were unchanged (Fig. 1). The sublines selected in chemosensitizer expressed significantly less *mdr1a* mRNA than the DNR-selected subline (EHR2/1.6V $P < 0.01$ and EHR2/1.6C $P < 0.05$; Fig. 2). Compared with EHR2, PGP increased 2.3-fold in the DNR plus CsA-selected subline, 7.8-fold in the DNR plus VER-selected subline, and 10.7-fold in the DNR-selected subline (Fig. 3). Thus, both chemosensitizer-coselected sublines expressed significantly less PGP than the DNR-selected subline ($P < 0.01$).

Six sublines were developed from the PGP-positive EHR2/DNR cell line (PGP 7.3 ± 1.1-fold increase compared with EHR2) by treatment with DNR or a combination of DNR and chemosensitizer. Compared with EHR2, all these sublines had increased *mdr1a* mRNA levels, whereas *mdr1b* mRNA levels were unchanged (Fig. 1), and the expression of *mdr1a* mRNA in the six sublines was not significantly different from that in EHR2/DNR (Fig. 2). Compared with EHR2/DNR, the expression of PGP increased significantly in the sublines treated with DNR alone ($P < 0.01$), whereas PGP did not increase ($P > 0.05$) in the DNR and chemosensitizer-selected sublines (Fig. 3). Furthermore, all DNR plus chemosensitizer-selected sublines had a lower expression of PGP than their DNR-selected counterparts, although this was not significant for EHR2/DNR/0.1V.

Expression *Mrp1* mRNA and *Mrp1*

Compared with EHR2, mRNA and protein levels of *Mrp1* did not change significantly in the selected sublines (Figs. 4 and 5).

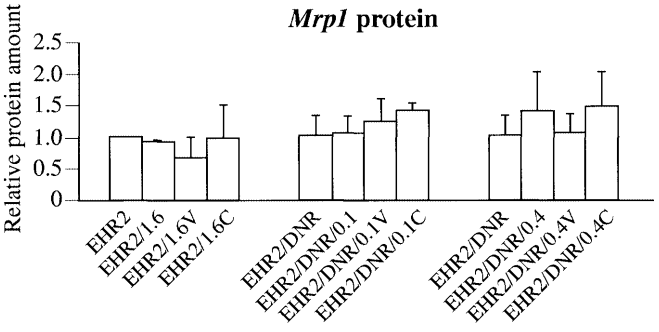

Fig. 5. Expression of *Mrp1* protein in the sensitive EHR2 cell line, in cell lines selected in DNR (EHR2/1.6, EHR2/DNR, EHR2/DNR/0.1, EHR2/DNR/0.4), in cell lines selected in DNR plus VER (EHR2/1.6V, EHR2/DNR/0.1V, EHR2/DNR/0.4V), and in cell lines selected in DNR plus CsA (EHR2/1.6C, EHR2/DNR/0.1C, EHR2/DNR/0.4C). *Bars* SD. In each series of experiments the sensitive cell line, EHR2, was used as a reference

Expression of topoisomerase IIα and β

In the DNR plus VER-selected subline EHR2/1.6V, TOPO IIα was reduced to one-third as compared to that in EHR2, whereas TOPO IIβ remained unchanged. Compared with EHR2, both TOPO IIα and β were unchanged in all other DNR- and DNR plus chemosensitizer-selected cell lines (data not shown).

Drug resistance

The clonogenic assay was used to determine DNR resistance. The DNR plus CsA-selected subline EHR2/1.6C was significantly less resistant (1.8-fold compared with EHR2) than the DNR-selected subline EHR2/1.6 (26.1-fold) and DNR plus VER-selected subline EHR2/1.6V (44.1-fold). In addition, resistance was significantly lower in the DNR plus CsA-selected sublines EHR2/DNR/0.1C (18.6-fold) and EHR2/DNR/0.4C (22.6-fold), as compared with the DNR-selected sublines

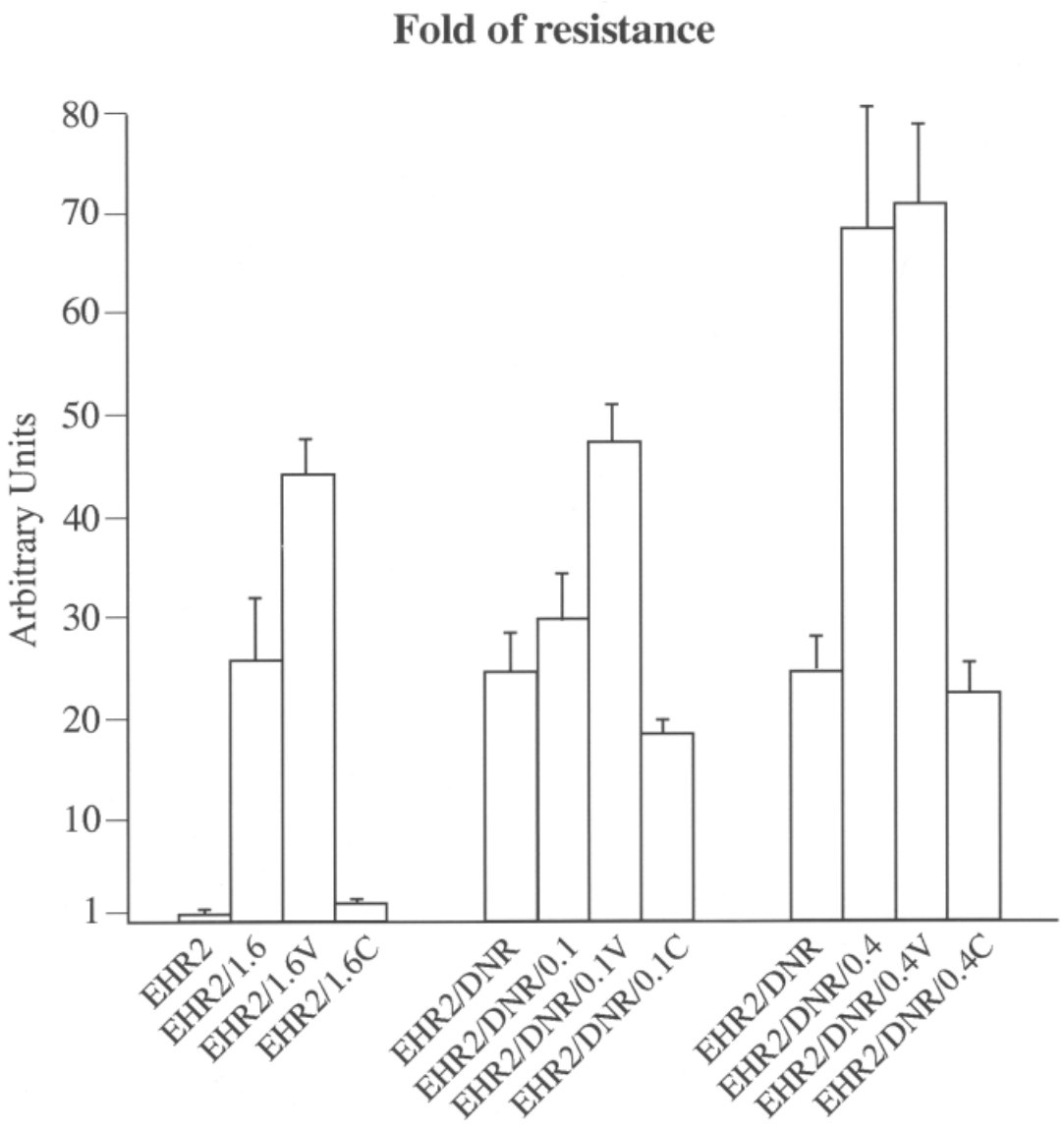

Fig. 6. Degrees of resistance of the sensitive EHR2 cell line [22], of cell lines selected in DNR (EHR2/1.6, EHR2/DNR, EHR2/DNR/0.1, EHR2/DNR/0.4), of cell lines selected in DNR plus VER (EHR2/1.6V, EHR2/DNR/0.1V, EHR2/DNR/0.4V), and of cell lines selected in DNR plus CsA (EHR2/1.6C, EHR2/DNR/0.1C, EHR2/DNR/0.4C). *Bars* SEM

EHR2/DNR/0.1 (29.8-fold) and EHR2/DNR/0.4) (68.4-fold) and their DNR plus VER-selected counterparts EHR2/DNR/0.1V (47.4-fold) and EHR2/DNR/0.4V (71.1-fold; Fig. 6).

Compared with the corresponding DNR-selected cell lines EHR2/1.6, EHR2/DNR/0.1 and EHR2/DNR/0.4, the resistance of the DNR plus VER-selected cell lines was increased in two (EHR2/1.6V and EHR2/DNR/0.1V; $P < 0.01$) and unchanged in one (EHR2/DNR/0.4V; $P > 0.05$; Fig. 6). In the EHR2/1.6V subline the increased resistance could be explained by the reduced expression of TOPO IIα.

Effect of VER on the cytotoxicity of DNR

Table 2 shows the relationship between the fold resistance of the different sublines and the degree of sensitization as a result of the presence of chemosensitizer. In all resistant cell lines, VER (3.1 μM) and CsA (1.0 μM) partly reversed the resistance. From a comparison of the DNR plus chemosensitizer-selected sublines with the DNR-selected sublines, it appeared that the chemosensitizers retained their ability to sensitize in all double-selected sublines.

Expression of PGP, *Mrp1* and accumulation of DNR

The correlation between PGP, *Mrp1*, and the accumulation of DNR at steady-state is shown in Table 3. Alterations in PGP expression probably explained the differences in accumulation of DNR in all sublines.

Table 2. Effect of VER and CsA on the cytotoxicity of DNR in Ehrlich ascites tumour cell lines selected in DNR or a combination of DNR and chemosensitizer. (The data shown are from six experiments)

Cell line	Relative resistance[a]	Fold reversal[b]	
		VER (3.1 μ*M*)	CsA (1.0 μ*M*)
EHR2	–	2.4[c]	1.9[c]
EHR2/1.6	26.1	10.5	11.5
EHR2/1.6V	44.1	29.9	19.4
EHR2/1.6C	1.8	3.8	3.4
EHR2/DNR/0.1	29.8	16.7	13.5
EHR2/DNR/0.1V	47.4	15.5	22.0
EHR2/DNR/0.1C	18.6	12.2	16.8
EHR2/DNR/0.4	68.4	22.4	26.0
EHR2/DNR/0.4V	71.1	11.1	17.5
EHR2/DNR/0.4C	22.6	8.8	19.5

[a]Relative resistance = ratio between the IC_{50} of the resistant cell line and that of EHR2
[b]Fold reversal = ratio between the IC_{50} in the absence and presence of chemosensitizer
[c]Reference 22

Table 3. Expression of PGP and *Mrp1* protein, and accumulation of DNR in Ehrlich ascites tumour cell lines selected in DNR or a combination of DNR and chemosensitizer. Values are means ± SD

Cell line	PGP (fold increase)	*Mrp1* (fold increase)	DNR accumulation (pmol/10^6 cells)
EHR2	–	–	1682 ± 12
EHR2/1.6	10.7 ± 1.0	0.9 ± 0.0	716 ± 18
EHR2/1.6 V	7.8 ± 0.8	0.7 ± 0.3	710 ± 3
EHR2/1.6C	2.3 ± 0.5	1.0 ± 0.5	1298 ± 37
EHR2/DNR	7.3 ± 1.1	1.0 ± 0.3	691 ± 10
EHR2/DNR/0.1	10.2 ± 1.9	1.0 ± 0.3	719 ± 23
EHR2/DNR/0.1V	8.9 ± 0.9	1.2 ± 0.4	507 ± 7
EHR2/DNR/0.1C	8.7 ± 1.5	1.4 ± 0.1	597 ± 93
EHR2/DNR/0.4	11.3 ± 1.5	1.4 ± 0.6	437 ± 19
EHR2/DNR/0.4V	8.7 ± 1.3	1.0 ± 0.3	473 ± 7
EHR2/DNR/0.4C	8.8 ± 1.4	1.4 ± 0.6	821 ± 22

Discussion

In order to investigate the influence of chemosensitizers on the emergence of the drug-resistant phenotype, we developed nine sublines of the sensitive Ehrlich ascites tumour cell line. Three sublines were treated with DNR, three with a combination of DNR and VER, and three with a combination of DNR and CsA.

Compared with treatment with DNR alone, repeated treatment with DNR and chemosensitizer in three of six double-selected cell lines resulted in a higher stochastic cell kill (tumour growth was significantly delayed). As VER and CsA have been reported to inhibit drug efflux from resistant cells [10], it is probable that this finding was mainly caused by an increase in the intracellular concentration of drug.

The drug-transporting class I genes in mice include *mdr1a* (*mdr3*) and *mdr1b* (*mdr1*). In the present study, the *mdr1a* mRNA levels were increased in all but one drug-selected subline, whereas *mdr1b* mRNA remained unchanged. Previously, Raymond et al. [23] have demonstrated increased expression of *mdr1a* mRNA in 7 of 12 independently derived MDR cell lines, of *mdr1b* mRNA in 3 of 12, and of both in two. Two sublines selected in doxorubicin had increased *mdr1a* mRNA levels only. In contrast, Zhou and Kuo [27] have shown increased expression of *mdr1b* 24 h after treatment of rat hepatoma cells with DNR. An explanation could be a selective growth advance of cells that overexpress *mdr1a* as compared to cells that overexpress *mdr1b*, as previously demonstrated by Lothstein et al. [18].

In the present study, VER seemed to suppress PGP in three individually selected sublines. Several authors have investigated the influence of VER on the drug-resistant phenotype with conflicting results [4, 13, 14, 19, 20]. In accordance with our results, Muller et al. [19] have reported a decreased expression of PGP in a human MDR leukaemia cell line treated with VER. Furthermore, Futscher et al. [13] have found that inclusion of VER during drug selection with doxorubicin prevents the emergence of PGP expression. The *mdr1a* mRNA level was significantly decreased in EHR2/1.6V selected from the sensitive cell line EHR2, whereas the *mdr1a* mRNA levels in the VER-selected sublines EHR2/DNR/0.1V and EHR2/DNR/0.4V were similar to the levels in the parent EHR2/DNR cell line and the DNR-selected counterpart. Because of its dynamic range, the RT-PCR was not able to show differences in cells with high levels of mRNA, explaining the findings in sublines EHR2/DNR/0.1V and EHR2/DNR/0.4V. Our results suggest that selection in VER decreases the expression of *mdr1a*. In accordance with this, Muller et al. have found decreased transcription of *mdr1* in leukaemic cells after treatment with VER [20]. These results, however, contradict those of Herzog et al. [14], who have reported an increase in the expression of *mdr1*/PGP in human colon carcinoma cell lines treated with different calcium channel blockers. In addition, Chaudhary and Roninson [4] have found that treatment with VER increases the expression of the *mdr1* gene in KG1 leukaemia cells, which spontaneously overexpress PGP.

All CsA-coselected cell lines (EHR2/1.6C, EHR2/0.1C, EHR2/DNR/0.4C) had a significantly lower expression of PGP than their DNR-selected counterparts. Furthermore, the amount of *mdr1a* mRNA was significantly decreased in subline EHR2/1.6C, whereas the *mdr1a* mRNA levels in the CsA-selected sublines EHR2/DNR/0.1C and EHR2/DNR/0.4C were similar to the levels in the EHR2/DNR subline and its DNR-selected counterpart. Like VER, selection in CsA seemed to decrease the expression of *mdr1a* and PGP. Studies focusing on the expression of PGP after the addition of CsA to chemotherapeutic treatment have shown conflicting results [2, 8, 14]. Dietel et al. [8] exposed PGP-positive Friend leukaemia cells to increasing doses of CsA at a constant level of doxorubicin and found that the PGP level in the resulting cell line was increased

fourfold. In addition, Herzog et al. [14] have reported increased expression of *mdr1*/PGP after incubation with CsA. These results, however, apparently contradict the findings of Beketic-Oreskovic et al. [2], who performed a Luria and Delbrück fluctuation analysis to determine the mutation rate and resistance mechanisms after a single-step selection with doxorubicin in the presence of the cyclosporin, PSC833. These authors concluded that coselection with cyclosporin reduces the mutation rate for doxorubicin-selected resistance tenfold and suppresses the emergence of *mdr1* mutants.

In the present study, we did not investigate the mutation rate or the transcription rate of *mdr1a*. Thus the exact mechanism by which the decreased *mdr1a* occurred is unknown. However, previous studies have shown that both mechanisms could account for the results as both have been described in cell lines selected in the presence of chemosensitizer [2, 20].

Chemosensitizers did not influence the mRNA or protein levels of *Mrp1*. In accordance with our results, Futscher et al. [13] and Abbaszadegan et al. [1] did not find increased expression of MRP1 in VER-coselected cell lines.

Only the subline EHR2/1.6 V developed changes in TOPO II, which suggests that VER may select for this resistance mechanism. In accordance with this suggestion, Futscher et al. [13] have demonstrated decreased TOPO II levels in myeloma cells selected in a combination of VER and doxorubicin. It should be emphasized, however, that this resistance mechanism has frequently been reported in MDR cell lines selected in cytostatic agent alone [12, 26]. As only one of three DNR plus VER-selected sublines developed changes in TOPO II, we suggest that neither VER nor CsA influences TOPO II.

The degrees of resistance were unchanged or increased in all the double-selected cell lines (EHR2/1.6V, EHR2/0.1V, EHR2/DNR/0.4V) in comparison with the DNR-selected cell lines. These results are consistent with the results of Formelli et al. [11], who reported an increased degree of resistance of melanoma cells selected in doxorubicin and VER. Decreased TOPO II could explain the findings in EHR2/1.6V. Furthermore, we did not investigate changes in TOPO II activity. A reasonable correlation between expression of PGP and accumulation of DNR was demonstrated, suggesting that PGP was the predominant drug-transporting resistance mechanism. Increased resistance following selection with DNR in the presence of VER could be a consequence of induction of other resistance mechanisms.

The chemosensitizers retained the ability to reverse the resistance in all double-selected cell lines. In contrast, Dietel et al. [8] have demonstrated resistance to CsA in Friend leukaemia cells exposed to CsA and doxorubicin. The continued ability of the chemosensitizers to modulate DNR resistance in the double-selected cells suggests that their affinity for PGP was unchanged.

It is remarkable that all DNR plus CsA-selected sublines were significantly less resistant than their DNR-selected counterparts. Clinical trials combining a chemosensitizer with cytostatic agent have failed to demonstrate conclusively that modulation of the MDR phenotype is of any benefit [6, 9]. A variety of strategies have been adopted. Most often, patients with drug-refractory disease have been retreated with the primary treatment combined with a chemosensitizer. The present study indicates that the use of CsA in initial treatment regimens could have the advantage of preventing the expression of PGP and the emergence of resistance in tumour types commonly known to develop increased resistance during or after the treatment. Examples include breast cancer, lymphoma, leukaemia, neuroblastoma, and multiple myeloma.

In conclusion, the present results indicate that inclusion of sensitizers during drug selection with DNR influences the drug-resistant phenotype. Selection in chemosensitizer seemed to decrease the expression of *mdr1a* and PGP. The chemosensitizers did not appear to influence *Mrp1* or TOPO II levels. Selection in CsA could retard the development of resistance.

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