Cancer Chemother Pharmacol (1997) 40: 150–158 © Springer-Verlag 1997

ORIGINAL ARTICLE

Sastry Gollapudi · Choong H. Kim · Bich-Ngoc Tran
Soni Sangha · Sudhir Gupta

Probenecid reverses multidrug resistance in multidrug resistance-associated protein-overexpressing HL60/AR and H69/AR cells but not in P-glycoprotein-overexpressing HL60/Tax and P388/ADR cells

Received: 1 April 1996 / Accepted: 25 October 1996

Abstract *Purpose*: To determine whether probenecid, an inhibitor of organic anion transport, is able to reverse multidrug resistance (MDR) through modulation of the drug transport function of MDR-associated protein (MRP) and P-glycoprotein (P-gP). *Methods*: Two MRP-overexpressing cell lines (HL60/AR and H69/AR) and two P-gP-overexpressing cell lines (HL60/Tax and P388/ADR) were cultured with different concentrations of daunorubicin (DNR) or vincristine (VCR) in the presence or absence of various concentrations of probenecid (0.01–10 m*M*). Drug sensitivity was determined using an MTT assay. DNR accumulation and subcellular distribution were determined by flow cytometry and confocal microscopy respectively. VCR accumulation was determined by scintillation spectrometry. *Results*: Probenecid, in a concentration-dependent manner, reversed resistance to DNR and VCR in HL60/AR and H69/AR tumor cell lines. This effect of probenecid on MDR was associated with an increased accumulation of DNR and VCR and correction of the altered subcellular distribution of DNR. The concentrations of probenecid that reversed MDR are clinically achievable in vivo. In contrast, probenecid did not reverse MDR in either HL60/Tax or P388/ADR tumor cell lines that overexpress P-gP. *Conclusion*: These results suggest that probenecid is an effective chemosensitizer of MRP-associated MDR tumor cells and is a potential candidate for clinical use to reverse MDR.

Key words MRP · Multidrug resistance · P-glycoprotein · Subcellular drug distribution · Drug transport

S. Gollapudi (✉) · C.H. Kim · B.-N. Tran · S. Sangha · S. Gupta
Division of Basic and Clinical Immunology, University of California, Irvine, CA 92717, USA
Tel. 714–824–5818; Fax 714–824–436

Introduction

The development of multidrug resistance (MDR) to anticancer agents by tumor cells is a major obstacle to the chemotherapeutic cure of cancer. Overexpression of P-glycoprotein (P-gP) or MDR-associated protein (MRP) has been shown to confer MDR in tumor cell lines [1–3]. Both P-gP and MRP are members of the ATP-binding cassette superfamily of membrane transport proteins [4, 5]. Increased expression of these membrane transport proteins is associated with decreased drug accumulation and/or altered subcellular drug distribution [2, 6–10]. A number of agents have been identified that reverse MDR in tumor cells that overexpress P-gP [11–15]. The majority of these chemosensitizing agents have been shown to reverse MDR by correcting the intracellular drug accumulation defect exhibited by MDR tumor cells [15–18]. Many of these modulators of MDR are less effective in reversing MDR associated with an overexpression of MRP [7, 19–21]. Recently, genestein, a tyrosine kinase inhibtor, and buthionine sulfoxamine, an inhibitor of glutathione synthesis, have been shown to reverse MDR in MRP, but not in P-gP-overexpressing cell lines [22–25]. The reversal of resistance by these modulators is seen at very high concentrations that are too toxic to use in humans. Currently, there is a need to identify agent(s) that can reverse MDR not mediated by P-gP and have no or minimal toxicity.

It has recently been demonstrated that leukotriene C4 (LTC4) and calcein, an organic anion, are transported by MRP [26, 27]. Probenecid, an inhibitor of organic anion transport, has been shown to decrease efflux of LTC4 and calcein [27–29]. Therefore, in this study we examined the in vitro chemosensitizing activity of probenecid in MDR cell lines that overexpress MRP, but lack P-gP expression (HL60/AR and H69/AR), and in cell lines that overexpress P-gP (HL60/Tax and murine leukemia P388/ADR). The present study demonstrated that probenecid, at clinically achievable serum concentrations, reversed MDR in HL60/AR and H69/AR cell lines but not in HL60/Tax and P388/

ADR cell lines. This would suggest that probenecid has a selective or preferential activity in MRP-overexpressing cells.

Materials and methods

Cell lines

MDR human myeloid leukemia cell lines (HL60/AR, HL60/Tax1000) and their parental drug-sensitive cell line (HL60) were obtained from Dr. Kapil Bhalla, University of South Carolina, Charleston, S.C. The MDR human small-cell lung carcinoma cell line (H69/AR) and drug-sensitive parental cell line H69 were kindly provided by Dr. Susan Cole, Queens University, Kingston, Ontario, Canada. These cell lines were maintained in suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 m*M* glutamine and 100 μg/ml each of penicillin and streptomycin. HL60/AR cells and H69/AR cells lack P-gP and its mRNA but overexpress the MRP gene and its product [5, 9, 30, 31]. The MDR murine leukemia cell line (P388/ADR) and its parental cell line (P388) were maintained in culture medium supplemented with 10^{-5} *M* 2-mercaptoethanol. HL60/Tax_{1000} and P388/ADR cells overexpress P-gP [19, 32]. We determined the expression of MRP and P-gp in MDR tumor cells at the message and protein levels by PCR and flow cytometry, respectively. The cross-resistant patterns of the MDR cells used in this study have been previously described [9, 14, 19, 33].

Polymerase chain reaction

MRP or *mdr*1 mRNAs were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using an RT-PCR kit from Perkin-Elmer (Forest City, Calif.) with specific primers. Briefly, total cellular RNA was extracted using the guanidinium isothiocyanate-phenol chloroform method [24], and cDNA was synthesized with 0.2 μg total cellular RNA and 2.5 μ*M* random hexamers in 20 μl solution containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* of each dNTP, and 2.5 units of reverse transcriptase for 30 min at room temperature. PCR was carried out for 35 cycles in 50 μl of a solution comprising 10 m*M* Tris-HCl (pH 8.3), 2 m*M* $MgCl_2$, and 0.2 m*M* of each dNTP, cDNA derived from 0.1 μg RNA, 1.25 unit of AmpliTaq DNA polymerase and 37.5 pmol of each primer of *mdr*1 or MRP. Each cycle of PCR consisted of 1 min of denaturation at 94 °C, 1 min of primer annealing at 60 °C and 2 min of extension at 72 °C. Primers specific for *mdr*1 (sense, 5′-CCCATCATTGCAATAGCAGG-3′; antisense, 5′-GTTCAAACTTCTGCTCCTGA-3′) and MRP (sense, 5′-TGAAGGACTTCGTGTCAGCC-3′; antisense, 5′-CGTCCATGATGGTGTTGAGCC-3′) yielded a 167 bp and a 242 bp DNA fragment, respectively. A 100-bp DNA ladder was used as a standard DNA size marker.

Flow cytometry

Expression of P-gp was determined with U1C2 (Immunotech, Westbrook, Me.) and C219 (Signet Laboratories, Dedham, Mass.) using a FACSan flow cytometer (Becton-Dickinson, San Jose, Calif.). HL60/AR, HL60/Tax and H69/AR cells (1×10^6) were incubated with 1 μg U1C2 or IgG2a isotypic control at 4 °C for 45 min. P388/ADR cells were permeabilized with 1 ml 70% methanol at −20 °C for 5 min, washed with cold phosphate-buffered saline (PBS) and incubated with 2 μg C219 or Ig2a isotypic control at 4 °C for 45 min. Cells were washed and counterstained with FITC-labeled goat antimouse Ig2a antibody. Cells were washed with PBS and the fluorescence intensity of 10 000 cells was determined using a FACScan flow cytometer. MRP expression was determined with MRPm6 monoclonal antibody (kindly provided by Prof. R.J. Scheper, Free University Hospital, Amsterdam). MDR tumor cells (1×10^6) were permeabilized and fixed with 1 ml 10% (v/v) FACS lysing solution (Becton-Dickinson), washed with PBS and incubated for 45 min with 0.5 μg MRPm6 or IgG1 isotypic control. Cells were washed and counterstained with FITC goat anti-mouse IgG1 antibody. Cells were washed and analyzed by FACSscan.

Drug sensitivity assay

Drug sensitivity was determined using a colorimetric MTT assay [34]. This assay is based on the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiozol-3-yl)-2,5-diphenyltetrazolium bromide] by a mitochondrial dehydrogenase in viable cells to a blue-colored formazan product that can be measured spectrometrically. The amount of formazan produced is proportional to the number of living cells. Cells were seeded in 96-well plates at 2×10^4/well and cultured in triplicate in the presence or absence of various concentrations of probenecid (0.1–0.5 m*M*) and with or without various concentrations of daunorubicin (DNR) (10^{-8} 5×10^{-5} *M*), or vincristine (VCR 0.1–1000 ng/ml). The cultures were incubated at 37 °C for 72 h, after which 50 μg of MTT was added to each well and the cultures incubated for an additional 4 h. The plates were centrifuged, the medium carefully removed, the formazan crystals solubilized with acid alcohol, and the plates read at 590 nm using an ELISA plate reader (Molecular Devices, Menlo Park, Calif.). The IC_{50} was defined as the concentration of drug that caused a 50% reduction in absorbance.

Drug accumulation

Intracellular accumulation of DNR and VCR was determined by flow cytometry and scintillation spectrometry, respectively [31]. Briefly, drug-sensitive and drug-resistant cells were incubated with 0.5 μ*M* DNR or 16 nM 3H-VCR (specific activity 6 Ci/mmol; Amersham, Arlington, Ill.) in the presence or absence of probenecid (0.1–0.5 m*M*) at 37 °C for 24 h. Accumulation of DNR was measured by flow cytometry, using a FACScan (Becton Dickinson) and the fluorescence intensity was recorded from histograms and the data expressed as mean fluorescence channel numbers (MFC). Cells incubated with 3H-VCR and probenecid were washed three times with ice-cold PBS and the cell pellets were dissolved in 2% sodium dodecyl sulfate solution. The cellular accumulation of VCR was determined using a scintillation counter and the data are expressed as picograms of VCR per 105 cells.

Subcellular distribution of DNR

Drug-sensitive (HL60 and H69) and drug-resistant (HL60/AR and H69/AR) cells were incubated with 0.5 μ*M* DNR in the presence or absence of probenecid (0.5 m*M*) for 24 h at 37 °C. Cells were deposited onto glass slides using a cytocentrifuge (SHANDON, Pittsburgh, Pa.), and mounted in cold RPMI-1640 medium using coverslips. The edges were sealed and the slides were kept on ice until analyzed. The subcellular distribution of DNR was assessed using a BioRad MRC600 laser scan confocal microscope.

Results

Expression of MRP and P-gp in MDR tumor cells

Figure 1 shows the expression of MRP at the protein level in the MDR cells. MDR HL60/AR, and H69/AR cells expressed MRP but not P-gP. In these cells, the P-gP message was also undetectable. In contrast, HL60/Tax and P388/ADR cells showed increased expression of P-gp, but not MRP (Fig. 1). Low levels of MRP message were detectable in HL60 cells but were undetectable in HL60/Tax cells (Fig. 2).

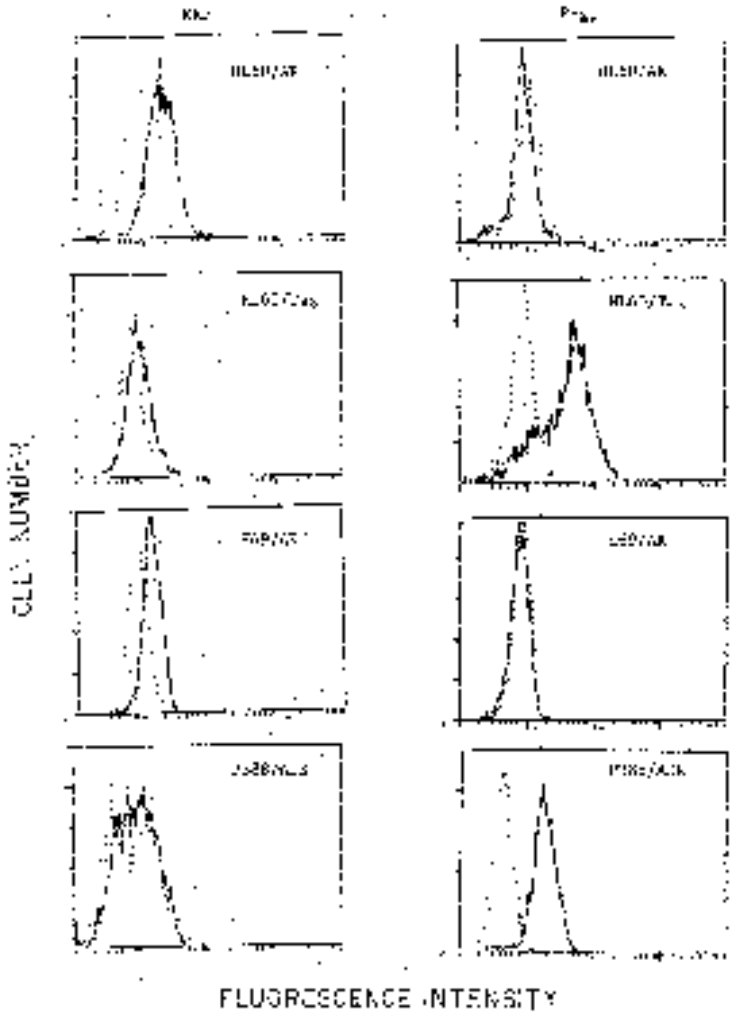

Fig. 1 Flow cytometric analysis of P-gp and MRP expression in MDR tumor cell lines. P-gP expression in HL60/AR and HL60/Tax and H69/AR cells was determined using monoclonal antibody U1C2. Monoclonal antibody C219 was used to determine the P-gP expression in P388/ADR cells. MRP expression was determined using monoclonal antibody MRPm6. *Dotted lines* represent staining with isotype control and solid lines represent staining with monoclonal antibody

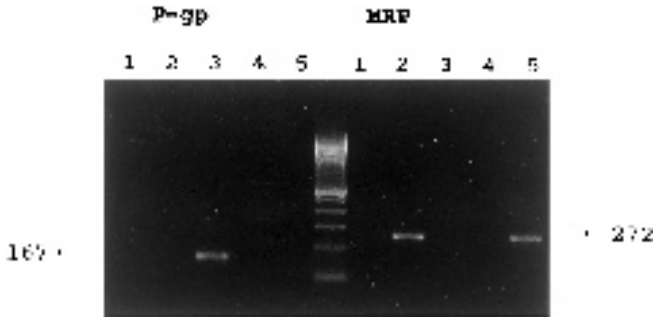

Fig. 2 Analysis of P-gp and MRP mRNA by RT/PCR in (*1*) HL60, (*2*) HL60/AR, (*3*) HL60/Tax, (*4*) H69 and (*5*) H69/AR cells. Total cellular RNA was extracted from each cell line, and P-gp and MRP mRNA were amplified by RT/PCR using specific primers as described in Materials and methods. The *centre lane* is a 100 bp DNA ladder

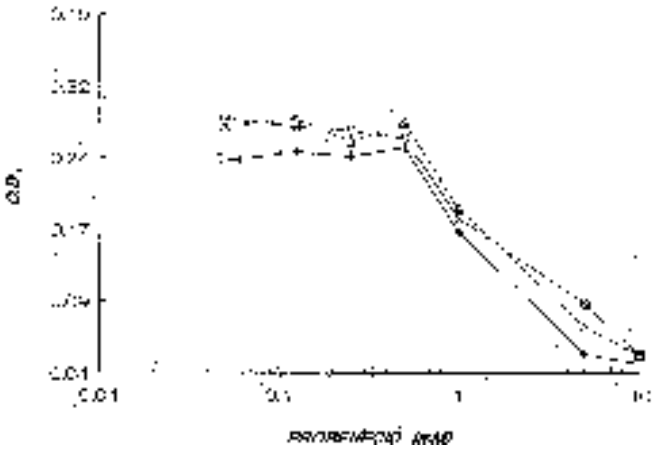

Fig. 3 Effect of probenecid on cell survival. HL60, HL60/AR and HL60/Tax and cells were cultured with or without the indicated concentrations of probenecid for 3 days. Cell survival was determined using an MTT assay. The OD values for HL60, HL60/AR and HL60/Tax cells cultured without probenecid were 0.210, 0.261 and 0.281, respectively. + HL60; –△– HL60/AR; –○– HL60/Tax

Probenecid reverses MDR in MRP but not PgP-expressing cell lines

The effect of different concentrations of probenecid on reversal of DNR and VCR resistance in MRP-expressing HL60/AR and H69/AR tumor cell lines is shown in Tables 1 and 2. Probenecid, in a concentration-dependent manner, increased the sensitivity and reversed the resistance to DNR and VCR in HL60/AR cells (Table 1) and in H69/AR cells (Table 2). The reversal of resistance by probenecid was

Table 1 Effect of probenecid on the drug sensitivity of HL60 and HL60/AR cells. Drug-sensitive and -resistant cells were incubated without or with the indicated concentrations of probenecid in the presence or absence of various concentrations of DNR or VCR. Drug sensitivity was measured using a 3-day MTT assay (*sensitization ratio* IC_{50} in the absence of probenecid IC_{50} in the presence of probenecid; *resistance factor* IC_{50} of resistant cells IC_{50} of sensitive cells

	Cell line	Probenecid concentration (m*M*)			
		0	0.1	0.25	0.5
Daunorubicin	HL60				
IC_{50} (μ*M*)		0.38 ± 0.19	0.27 ± 0.14	0.23 ± 0.10	0.26 ± 0.13
Sensitization ratio			1.41	1.65	1.46
IC_{50} (μ*M*)	HL60/AR	2.53 ± 0.33	1.75 ± 0.31*	0.86 ± 0.05**	0.56 ± 0.19**
Sensitization ratio			1.45	2.94	4.52
Resistance factor		6.66	6.48	3.74	2.15
Vincristine					
IC_{50} (n*M*)	HL60	3.5 ± 0.9	3.6 ± 1.0	3.0 ± 1.0	3.4 ± 0.7
Sensitization ratio			0.97	1.17	1.03
IC_{50} (n*M*)	HL60/AR	24.0 ± 0.5	16.5 ± 0.5**	4.0 ± 0.4**	2.7 ± 1.2**
Sensitization ratio			1.45	6.00	8.89
Resistance factor		6.86	4.58	1.33	0.79

* $P \leq 0.05$, ** $P \leq 0.01$, vs IC_{50} values in the absence of probenecid

Table 2 Effect of probenecid on the drug sensitivity of H69 and H69/AR cells. Drug-sensitive and -resistant cells were incubated without or with the indicated concentrations of probenecid in the presence or absence of various concentrations of DNR or VCR. Drug sensitivity was measured using a 3-day MTT assay (*sensitization ratio* IC_{50} in the absence of probenecid IC_{50} in the presence of probenecid; *resistance factor* IC_{50} of resistant cells IC_{50} of sensitive cells

	Cell line	Probenecid concentration (m*M*)			
		0	0.1	0.25	0.5
Daunorubicin					
IC_{50} (μ*M*)	H69	1.5 ± 0.60	1.4 ± 0.2	1.2 ± 0.30	1.5 ± 0.80
Sensitization ratio			1.07	1.25	1.0
IC_{50} (μ*M*)	H69/AR	24.5 ± 0.33	8.4 ± 2.90*	7.2 ± 0.28*	4.1 ± 0.19*
Sensitization ratio			2.9	3.4	5.98
Resistance factor		16.33	6.0	6.0	2.7
Vincristine					
IC_{50} (n*M*)	H69	9.9 ± 0.4	10.3 ± 2.0	9.4 ± 2.2	7.5 ± 1.7
Sensitization ratio			0.96	1.05	1.32
IC_{50} (n*M*)	H69/AR	573.0 ± 64	64.7 ± 11*	50.6 ± 9*	44.0 ± 2*
Sensitization ratio			8.85	11.30	13.00
Resistant factor		57.9	6.28	5.4	5.9

* $P \leq 0.01$, vs IC_{50} values in the absence of probenecid

almost complete in HL60/AR cells, but was incomplete in H69/AR cells. Probenecid had no significant ($P \geq 0.05$) effect on the sensitivity of HL60 cells to DNR and VCR. Probenecid did not alter the sensitivity of P-gP-expressing HL60/Tax and P388/ADR cells or of their parental cell lines HL60 and P388 cells to either DNR or VCR (Tables 3 and 4).

The viability of the cells cultured with various concentrations of probenecid is shown Fig. 3. Probenecid at concentrations lower than 1 m*M* was not toxic to tumor cells, but at concentrations above 1 m*M* it was toxic to cells exposed continuously. Experiments were therefore performed to determine whether pulse exposure to high concentrations of probencid could revese MDR. In these experiments, HL60 and HL60/AR cells were exposed to different concentrations of DNR in the presence or absence of 10 m*M* probenecid for 1 h. After exposure the cells were washed to remove the drugs and cultured for 3 days in the absence of DNR and probenecid. It can be seen from the results in Table 5 that pulse exposure of cells to probenecid and DNR resulted in partial reversal of MDR in HL60/AR cells.

Probenecid increases intracellular drug accumulation and alters subcellular distribution of DNR

The effect of probenecid on drug accumulation and subcellular drug distribution in HL60/AR and H69/AR cells was studied to investigate the possibilty that probenecid interferes with the function of MRP. Overexpression of MRP has been reported to be associated with reduced drug accumulation (in HL60/AR) and alterations in subcellular drug distribution (in HL60/AR and H69/AR cells) [6–10, 31]. Therefore, the effect of probenecid on drug accumulation was studied in HL60/AR cells and drug distribution in both the cell lines. Drug accumulation in HL60 and HL60/AR cells following a 24-h exposure to 0.5 μ*M* DNR or 15 ng 3H-VCR with or without various concentrations of proben-

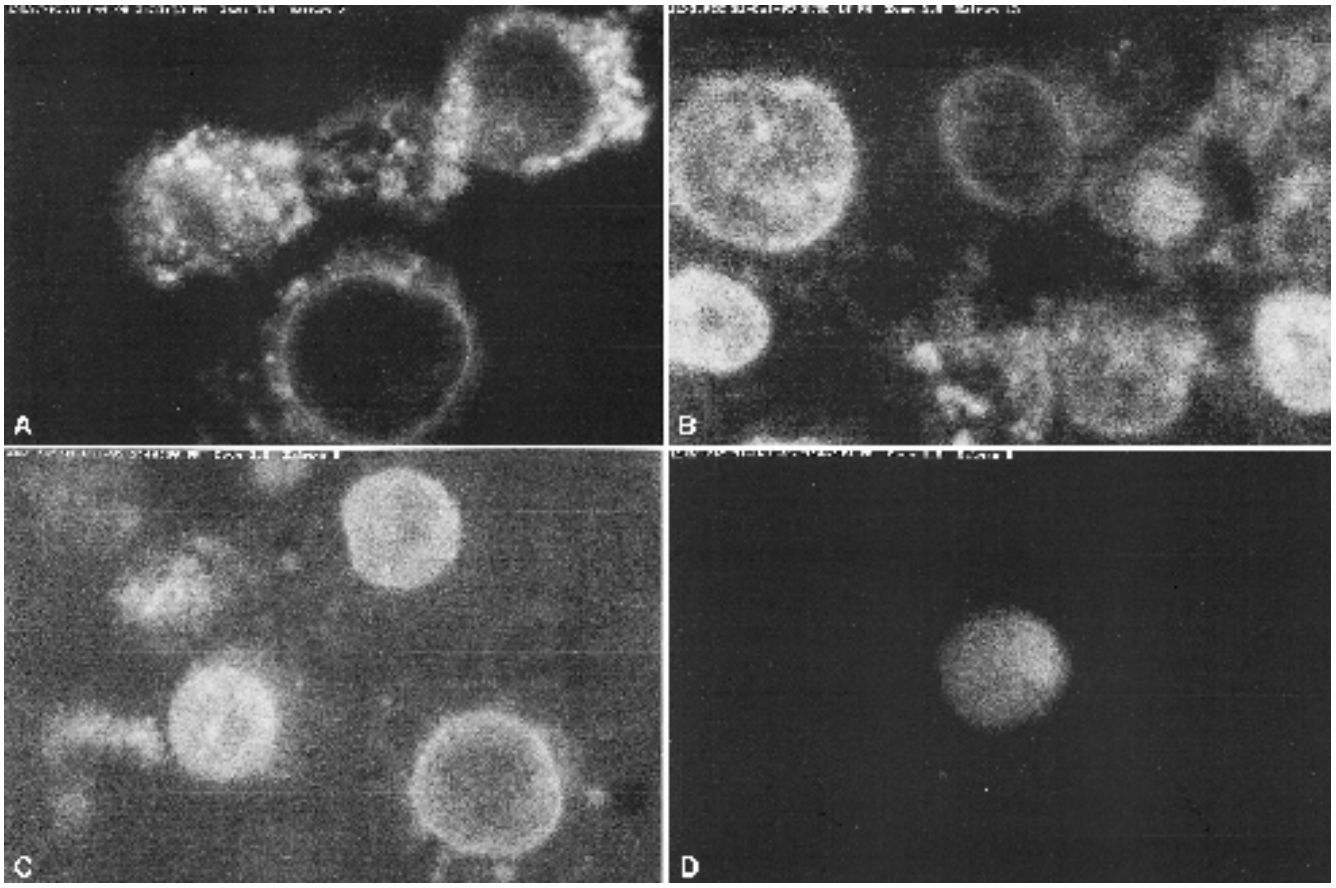

Fig. 4A–D Confocal microscopy of the subcellular distribution of DNR in untreated (**A**) and probenecid-treated (**B**) HL60/AR cells and in untreated (**C**) and probenecid-treated (**D**) HL60 cells

ecid is shown Table 6. Probenecid at concentrations of 0.1 m*M* and above caused significant increases in DNR and VCR accumulation in HL60/AR cells. The subcellular distribution of DNR in drug-resistant and -sensitive parental tumor cell lines exposed to 0.5 μ*M* DNR, in the presence or absence of 0.5 m*M* probenecid for 24 h is shown in Figs. 4

Table 3 Effect of probenecid on the sensitivity of HL60/Tax cells to DNR and VCR. Drug-sensitive and -resistant cells were incubated without or with the indicated concentrations of probenecid in the presence or absence of various concentrations of DNR or VCR. Drug sensitivity was measured using a 3-day MTT assay

Probenecid (m*M*)	DNR (IC_{50} in μ*M*)		VCR (IC_{50} in n*M*)	
	HL60	HL60/Tax	HL60	HL60/Tax
0	0.38 ± 0.19	1.97 ± 0.46	3.5 ± 0.9	2000 ± 400
0.1	0.27 ± 0.14	2.93 ± 1.32	3.6 ± 1.0	2250 ± 212
0.25	0.23 ± 0.10	3.2 ± 1.14	3.0 ± 1.0	1700 ± 360
0.5	0.26 ± 0.13	3.35 ± 1.08	3.4 ± 0.70	2200 ± 264

Table 4 Effect of probenecid on the sensitivity of P388 and P388/ADR cells to DNR and VCR. Drug-sensitive and -resistant cells were incubated without or with the indicated concentrations of probenecid in the presence or absence of various concentrations of DNR or VCR. Drug sensitivity was measured using a 3-day MTT assay

Probenecid (m*M*)	DNR (IC_{50} in μ*M*)		VCR (IC_{50} in n*M*)	
	P388	P388/ADR	P388	P388/ADR
0	0.11 ± 0.03	1.78 ± 0.22	0.38 ± 0.06	36.8 ± 6.5
0.1	0.11 ± 0.04	1.45 ± 0.17	0.30 ± 0.06	33.6 ± 1.5
0.25	0.10 ± 0.04	1.53 ± 0.19	0.30 ± 0.02	30.7 ± 8.5
0.5	0.09 ± 0.02	1.65 ± 0.26	0.27 ± 0.05	32.0 ± 4.4

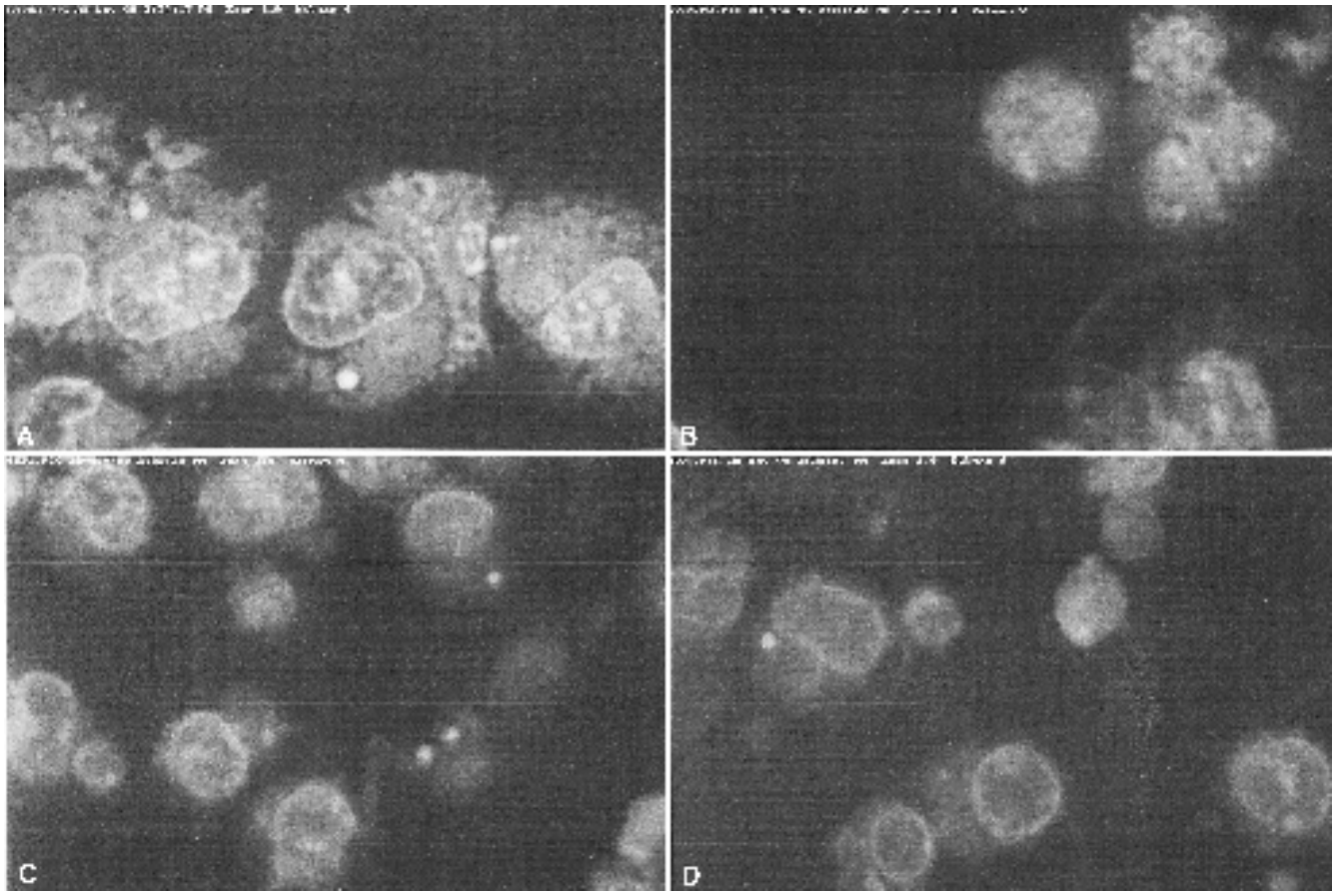

Fig. 5A–D Confocal microscopy of the subcellular distribution of DNR in untreated (**A**) and probenecid-treated (**B**) H69/AR cells and in untreated (**C**) and probenecid-treated (**D**) H69 cells

and 5. As expected, the DNR staining pattern of drug resistant cells was quite dissimilar from that seen in parental drug-sensitive cells. In HL60/AR cells (Fig. 4A) DNR was distributed in perinuclear regions with little or no nuclear accumulation. In the presence of probenecid, DNR accumulated in the nucleus of HL60/AR cells. This pattern

Table 5 Effect of probenecid on the IC_{50} value (μ*M*) of DNR in HL60 and HL60/AR cells. Drug-sensitive and -resistant cells were incubated for 1 h witu various concentrations of DNR in the presence or absence of 10 m*M* probenecid, washed to remove the drugs and cultured for 3 days. Drug sensitivity was measured using an MTT assay

Cells preexposed to	HL60	HL60/AR
DNR	0.62 ± 0.03	3.6 ± 1.1
DNR + probenecid	0.61 ± 0.05	1.5 ± 0.07

Table 6 Effect of probenecid on DNR and VCR accumulation in HL60 and HL60/AR cells. HL60 and HL60/AR cells were incubated without or with the indicated concentrations of probenecid in the presence of 0.5 μ*M* DNR or 15 ng/ml ^{3}H-VCR for 24 h at 37 °C. DNR accumulation was measured using a FACScan flow cytometer, and the results from a representative of two separate experiments each done in triplicate are expressed as fluorescence channel number (mean ± SD). VCR accumulation was determined by scintillation spectrometer, and results shown are the mean of two separate experiments

Probenecid (m*M*)	DNR (fluorescence intensity)		VCR (picograms/10^5 cells)	
	HL60	HL60/AR	HL60	HI60/AR
0	294 ± 3	174 ± 5	34	25.5
0.01	294 ± 2	174 ± 3	ND	ND
0.05	303 ± 5	175 ± 1	ND	ND
0.1	287 ± 2	227 ± 2	25	40.3
0.25	283 ± 3	248 ± 4	29	46.7
0.5	278 ± 4	251 ± 5	33	60.9

Table 7 Effect of probenecid on DNR accumulation. HL60 and HL60/AR cells were incubated with or without the indicated concentrations of probenecid in the presence of 4 μg/ml DNR at 37 °C for 1 h. DNR accumulation was measured using a FACScan flow cytometer, and the results are expressed as fluorescence channel number (mean ± SD)

Probenecid (m*M*)	HL60	HL60/AR
0	1104 ± 20	928 ± 19
0.1	1088 ± 31	941 ± 7
0.25	1106 ± 16	946 ± 16
0.5	1075 ± 61	995 ± 53
1.0	1172 ± 26	1090 ± 41
5.0	1117 ± 54	1080 ± 20
10.0	1133 ± 33	1110 ± 20

of DNR distribution was similar to that observed in parental drug-sensitive HL60 cells (Fig. 4C). Probenecid had no effect on the distribution of DNR in HL60 cells (Fig. 4D). In H69/AR cells, particulate nuclear and cytoplasmic staining was seen (Fig. 5A), whereas in H69 cells diffuse nuclear staining was observed (Fig. 5C). The staining pattern of H69/AR cells treated with probenecid (Fig. 5B) was similar to that of the parental H69 line showing diffuse nuclear staining (Fig. 5C). H69 cells treated with probenecid showed no change in the subcellular distribution of DNR (Fig. 5D).

The data in Table 7 shows DNR accumulation in HL60 and HL60/AR cells following a 1-h exposure to 4 μg/ml DNR and various concentrations of probenecid. Probenecid at a concentration of 10 m*M* completely corrected the drug accumulation defect in HL60/AR cells, but at low concentrations (0.1 – 0.5 m*M*) had no significant effect on DNR accumulation in 1-h assay.

Discussion

In this investigation, we demonstrated that probenecid is an effective chemosensitizer for MDR cells that overexpress MRP. The concentrations of probenecid (0.1 – 0.5 m*M*) that reversed MRP-mediated MDR are readily achievable in vivo. Following a single oral dose of 200 mg of probenecid, the peak plasma level in humans is 0.7 m*M* [35].

The precise mechanism(s) by which probenecid reverses MDR remain(s) unclear. However, the observation that probenecid reversed MDR in MRP-overexpressing but not P-gP-overexpressing cell lines suggests that the chemosensitizing effect of probenecid is linked to the overexpression of MRP. MRP is a member of the ATP-binding cassette superfamily of membrane transport proteins. It has been suggested that MRP plays a role in reduced drug accumulation [6], altered intracellular distribution of anthracyclines [7 – 10], and resistance to natural product chemotherapeutic agents and to anions such as arsenite and antimonials [36]. In addition, MRP has been shown to facilitate the transport of LTC4 [26] and calcein [27]. Probenecid, in the concentration range 0.7 – 1 m*M*, has been shown to inhibit the transport of LTC4 [25, 28, 29] and calcein [27] in MRP-overexpressing cells. Recently, Evers at al. [37] have reported that probenecid (2.5 m*M* and 10 m*M*), increases DNR accumulation in polarized kidney epithelial cells transfected with an MRP cDNA vector. In this study we showed that the effect of probenicid on DNR accumulation in HL60/AR cells is time- and dose-dependent; high concentrations of probenecid (1 – 10 m*M*) reversed the drug accumulation deficit in a short period of time (1 h), whereas low concentrations of probenecid (0.125 – 0.5 m*M*) increased DNR accumulation after a prolonged (24-h) incubation. A possible explanation for these findings is that a critical intracellular concentration of probenecid may be required to exert its effect on the drug transport activity of MRP. This threshhold level may be achieved in a short period of time with high concentrations of probenecid and longer incubation times may be required to achieve this threshhold level with low probenecid concentrations. Whether probenecid inhibits transport function of MRP by directly interacting with drug binding sites on MRP or indirectly by altering cellular levels of glutathione or ATP remains to be investigated. It should be mentioned that levels of probenecid in the range 1 – 10 m*M* are not achievable clinically. We found that continuous exposure to concentrations of probenecid in this range was very toxic to the cells used in this study.

Gekeler et al. [38] have shown that MK571, an LTC4 receptor antagonist, similar to the effect of probenecid observed in the present study, reverses anthracycline and VCR resistance in GLC4/ADR cells. However the effect of MK571 on drug transport was not reported. Manzano et al. [39], Zaman et al. [24] and Lutzky et al. [25] have shown that agents that deplete cellular glutathione levels, buthionine sulfaxamine (BSO), acrolein and chloroacetaldehyde, reverse MRP-mediated, but not P-gP-mediated, MDR by correcting the drug accumulation deficit. Rhodes et al. [40] have shown that bafilomycin A and 7-chloro-4-nitrobenza-2-oxa-1-3-diazole, inhibitors of H^+-ATPases, increase drug accumulation and modify subcellular distribution in MRP-overexpressing COR-L23/R cells. However, these agents were shown to be ineffective in reversing drug resistance. In this study we demonstrated that pulse exposure of HL60/AR cells to DNR plus probenecid completely corrected the drug accumulation defect but partially reversed DNR resistance. We also showed that probenecid corrected the altered subcellular drug distributions in H69/AR cells, but did not completely reverse MDR in this cell line. Our results, together with those of Cole et al. [36] and Rhodes et al. [40], demonstrate that there is no correlation between drug accumulation, subcellular drug distribution, and drug sensitivity. This discrepancy between correction of drug dis-

tribution abnormality by probenecid and its chemosensitizing activity is not unexpected since MDR cells have been shown to have additional resistance mechanisms such as reduced levels of topoisomerase α and β [41], alterations in the levels of glutathione [24, 25], increased expression of annexin II [42] and changes in the intracellular pH [43]. It is possible that these mechanisms may be insensitive to the modulatory effects of probenecid.

Regardless of the mechanisms, our findings suggest that probenecid is a candidate for clinical trials of its potential to reverse MDR associated with the overexpression of MRP.

References

1. Ueda K, Carderelli C, Gottesman MM, Pastan I (1987) Expression of a full length cDNA for the human MDR1 gene confers resistance to colchicine, doxorubin and vinblastine. Proc Natl Acad Sci USA 84:3004–3008
2. Endicott J, Ling V (1989) The biochemistry of P-glycoprotein mediated multidrug resistance. Annu Rev Biochem 58:131–171
3. Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley RG (1994) Overexpression of multidrug resistance associated protein (MRP) increases resistance to natural product drugs. Cancer Res 54:357–361
4. Juranka PF, Zastawny RL, Ling V (1989) P-glycoprotein: multidrug resistance and a superfamily of membrane associated transport proteins. FASEB J 3:2583–2592
5. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz AU, Duncan AM, Deeley RG (1992) Overexpression of a transporter gene in a multidrug resistant human cancer cell line. Science 258:1650–1654
6. Zaman GJR, Flens MJ, Van Leusden MR, De Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Bass F, Broxterman HJ, Borst P (1994) The human multidrug resistance asociated protein MRP is a plasma membrane drug efflux pump. Proc Natl Acad Sci USA 91:8822–8826
7. Barrand MA, Rhodes T, Center MS, Twentyman PR (1992) Chemosensitisation and drug accumulation effects of cyclosporin A, PSC833 and verapamil in human MDR large cell lung cancer cells expressing a 190-KD membrane distinct from P-glycoprotein. Eur J Cancer 29A:408–415
8. Marquardt D, Center MS (1992) Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: evidence for nuclear drug accumulation and redistribution in resistant cells. Cancer Res 52:3157–3163
9. Hindenburg AA, Gervasoni JE, Krishna S, Stewart VJ, Rosado M, Lutzky J, Bhalla K, Baker MA, Taub RN (1989) Intracellular distribution and pharmacokinetics of daunorubicin in anthracycline-sensitive and -resistant HL60 cells. Cancer Res 49: 4607–4614
10. Gervasoni JE, Fields SJ, Krishna S, Baker MA, Rosado M, Thuraisamy K, Hindenburg AA, Taub RN (1991) Subcellular distribution of daunorubicin in P-glycoprotein positive and -negative drug resistant cell lines using laser assisted-confocal microscopy. Cancer Res 51:4955–4963
11. Tsuruo T, Iido H, Tsukagoshi SH, Sakurai Y (1981) Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 41:1667–1672
12. Zamora JM, Beck WT (1986) Chloroquine enhancement of anticancer drug cytotoxicity in multiple drug resistant human leukemic cells. Biochem Pharmacol 35:4303–4310
13. Slater LM, Sweet P, Stupecky M, Gupta S (1986) Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia in vitro. J Clin Invest 77:1405–1408
14. Gupta S, Kim J, Gollapudi S (1991) Reversal of daunorubicin resistance in P388/ADR cells by itraconozole. J Clin Invest 87:1467–1469
15. Ford JM, Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev 42:155–199
16. Safa AR, Glover CJ, Sewell JL, Meyers MB, Biedler JL, Felsted RL (1987) Identification of the multidrug resistance related membrane glycoprotein as an acceptor for calcium channel blockers. J Biol Chem 262:7884–7888
17. Cornwell MM, Pastan I, Gottesman MM (1987) Certain calcium channel blockers bind specifically to multidrug resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem 262:2166–2170
18. Saeki T, Ueda K, Tanigawara Y, Hori R, Komano T (1993) Human P-glycoprotein transports cyclosporin A and FK-506. J Biol Chem 268:6077–6080
19. Gollapudi S, Gupta S (1992) Lack of reversal of daunorubicin resistance in HL60/AR cells by cyclosporin A. Anticancer Res 12:2127–2132
20. Cole SPC (1992) Multidrug resistance in small lung cancer. Can J Physiol Pharmacol 70:313–329
21. Cole SP, Downes HF, Slovak ML (1989) Effect of calcium antagonists on the chemosensitivity of two multidrug-resistant human tumor cell lines which do not overexpress P-glycoprotein. Br J Cancer 59:42–46
23. Versantvoort CHM, Schuurhuis GJ, Pinedo HM, Eakman CA, Kuiper CM, Lankelma J, Broxterman HJ (1993) Genestein modulates the decreased drug accumulation in non-P-glycoprotein-mediated multidrug resistant tumor cells. Br J Cancer 68:939–946
24. Zaman GJ, Lankelma J, Van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RP, Baas F, Borst P (1995) Role of glutathione in the exports of compounds from the cells by the multidrug resistance associated protein. Proc Natl AcadSci (USA) 92:7690–7694
25. Lutzky A, Astor MB,Taub RN, Baker MA, Bhalla K, Gervosani JR, Rosado MR, Stewart M, Krishna S, Hindenburg AA (1989) Role of glutathione and dependent enzymes in anthracycline resistant Hl60/AR cells. Cancer Res 49:4120–4125
26. Leier I, Jedlitscky G, Buchholz U, Cole SP, Deeley RG, Keppler D (1994) The MRP gene encodes an ATP dependent export pump for leukotriene C4 and structurally related conjugates. J Biol Chem 264:27807–27810
27. Feller N, Broxterman HJ, Wahrer DCR, Pinedo HM (1995) ATP-dependent efflux of calcein by multidrug resistance protein (MRP). No inhibition by intracelluar glutathione depletion. FEBS Lett 368:385–388
28. Leier I, Jedlitscky G, Buchholz U, Keppler D (1994) Characterization of the ATP-dependent leukotriene C4 export carrier in mastocytoma cells. Eur J Biochem 220:599–606
29. Lam BK, Xu K, Atkins MB, Austen KF (1992) Leukotriene C4 uses a probenecid sensitive export carrier that does not recognize leukotriene B4. Proc Natl Acad Sci USA 89:11598–11602
30. Krishnamachary N, Center MS (1993) The MRP gene associated with a non P-glycoprotein multidrug resistance encodes a 190 KDa membrane bound glycoprotein. Cancer Res 53:3658–3661
31. Gollapudi S, Thadepalli F, Kim CH, Gupta S (1995) Difloxacin reverses multidrug resistance in HL60/AR cells that overexpress multidrug resistance related protein (MRP) gene. Oncol Res 7:73–85
32. Bhalla K, Huang Y, Tang C, Self S, Ray S, Mahoney M, Bonnathpur V, Tourkina E, Ibrado A, Bullock G, Willingham MC (1994) Characterization of human myeloid leukemia cell line highly resistant to taxol. Leukemia 8:465–475
33. Mirski SEL, Gerlach JH, Cole SPC (1987) Multidrug resistance in a small cell lung cancer cell line selected in adriamycin. Cancer Res 47:2594–2598
34. Mossman T (1983) Rapid colorometric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
35. Gilman AG, Rall TW, Nies AS, Taylor P (eds) (1990) The pharmacological basis of therapeutics (8th edn). Pergamon Press, New York, p 1703

36. Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 54:5902–5910
37. Evers R, Zaman GJR, Van Deemeter L, Jansen H, Calafat J, Oomen LCJM, Oude Elferink RPJ, Borst P, Schinkel AH (1996) Basolateral localization of export activity of the multidrug resistance associated protein in polarized pig kidney cells. J Clin Invest 97:1211–1218
38. Gekeler V, Ise W, Sanders KH, Ulrich W, Beck J (1995) The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP-associated multidrug resistance. Biochem Biophys Res Comm 208:345–352
39. Manzano RG, Wright KA, Twentyman PR (1996) Modulation by acrolein and chloroacetaldehyde of multidrug resisatnce mediated by the multidrug resisatnce associated protein. Clin Cancer Res 2:1321–1326
40. Rhodes T, Barrand MA, Twentyman PR (1994) Modification by brefeldin A, bafilomycin A1 and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD) of cellular accumulation and intracellular distribution of anthracyclines in the non-P-glycoprotein mediated multidrug resistatant cell line COR-L23/R. Brit J Cancer 70:60–66
41. Cole SPC, Chanda ER, Dickie FP, Gerlach JH, Mirski SEL (1991) Non-P-glycoprotein mediated multidrug resistance in small cell lung cancer cell lines: evidence for a decreased susceptibilty to drug induced DNA damage and reduced levels of topoisomerase II. Cancer Res 51:3345–3352
42. Cole SPC, Pinkoski MJ, Bhardwaj G, Deeley RG (1992) Elevated expression of annexin (lipocortin II, p36) in a multidrug resistant small cell lung cancer cell line. Br J Cancer 65:498–502
43. Schneider E, Altenberg GA, Cowan KH (1994) Increased intracellular pH as a possible mechanism of MRP mediated multidrug resistance in the human mammary carcinoma cell line MCF7/VP (abstract). Proc Am Assoc Can Res 36:325