CORRELATION BETWEEN FUNCTIONAL AND MOLECULAR ANALYSIS OF *mdr1* P-GLYCOPROTEIN IN HUMAN SOLID-TUMOR XENOGRAPTS

H.J. BROXTERMAN^{1,3}, N. FELLER¹, C.M. KUIPER¹, E. BOVEN¹, C.H.M. VERSANTVOORT¹, T. TEERLINK², H.M. PINEDO¹ and J. LANKELMA¹

Departments of ¹Medical Oncology and ²Clinical Chemistry, Free University Hospital, Amsterdam, The Netherlands.

The contribution of P-glycoprotein (Pgp) to multidrug resistance in human solid tumors is generally estimated from bulk mRNA measurements or immunohistochemistry, while direct measurement of the effect of Pgp on intracellular drug concentrations has not been pursued. We investigated the feasibility and sensitivity of a method for probing Pgp-mediated drug transport in cells isolated from solid tumors, using xenograft models. Human tumor xenografts (XG) were grown by s.c. injection of Pgp-expressing cell lines 2780^{AD}, BRO/*mdr1* and KB8-5. Tumor uptake of doxorubicin (DOX) after administration of DOX to the mice was determined. XG from untreated mice were enzymatically dissociated. The effect of the Pgp modulator bepridil on steady-state cellular daunorubicin (DNR) and vincristine (VCR) accumulation and chemosensitivity of these XG cells was compared with its effects in the cell lines (CL). *mdr1* mRNA and Pgp (by flow cytometry) were measured. Also, the dependence on intracellular ATP concentration, [ATP]_i, of the modulator effect was determined in intact KB8-5 cells. The results showed that i.v. administration of DOX to the mice led to lower DOX levels in the Pgp-expressing XG than in the "sensitive" XG, suggesting the presence of an *in vivo* functional Pgp in these XG tumor models. Dissociated, viable XG cells appeared to have ATP levels sufficient to sustain Pgp-ATPase-coupled drug transport. This was inferred from experiments using KB8-5 CL, which showed half-maximal inhibition of DNR transport at an [ATP]_i of 1 to 2 mM. The effect of bepridil on DNR and VCR accumulation and chemosensitivity in the XG cells was in accordance with the XG expression of *mdr1*/Pgp. In KB8-5 XG cells, Pgp function was hardly detectable, in accordance with decreased *mdr1*/Pgp expression *in vivo*. In conclusion, Pgp activity can be determined in freshly dissociated XG human tumor cells. The results obtained with the more necrotic KB8-5 XG may represent some of the interpretation problems arising when low levels of Pgp expression occur within a heterogeneous cell population, such as may be expected in clinical human tumors. Also our results indicate that Pgp activity may be impaired *in vivo* at [ATP]_i below 2 mM, which are realistic values for human solid tumors.

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Since one of several factors limiting effective chemotherapy of cancer may be the occurrence of cellular drug resistance, it would be a considerable improvement if the sensitivity or resistance of a patient's tumor cells to the available cytostatic agents could be predicted. For this purpose, attempts have been made to culture primary human tumor cells to establish their chemosensitivity profile. Some, but limited success has been gained, e.g., by clonogenic assays or ³H-thymidine incorporation. Major problems, mainly related to the lack of human tumor-cell growth *in vitro*, have limited the wider application of these techniques (Von Hoff, 1988; Weisenthal, 1992).

With the availability of probes for specific genes mediating drug resistance, primarily *mdr1*/P-glycoprotein (*mdr1*/Pgp), the perspective for drug-resistance testing gained new impulses. Gene probes and monoclonal antibodies for *mdr1*/Pgp have been used to assess the incidence of the multidrug-resistant (MDR) phenotype in clinical tumor specimens (Fojo *et al.*, 1987; Van der Valk *et al.*, 1990), and correlations with clinical response have been made in certain tumor types (Chan *et al.*, 1990).

A more stringent test for involvement of the MDR phenotype in clinical drug resistance would require assessment of the impact of Pgp-mediated active drug transport on the intracellu-

lar drug concentration in tumor cells. This would avoid interpretation problems, caused by the fact that neither *mdr1* mRNA nor Pgp protein levels take into account the effect of post-translational modifications, in particular the phosphorylation state of Pgp, which may alter Pgp function (Chambers *et al.*, 1990). Furthermore, even in cells with the same number of active Pgp drug-transporter molecules, the net effect of Pgp on cellular drug pharmacokinetics will be different if the passive drug uptake through the plasma membrane differs (Lampidis *et al.*, 1989; Spoelstra *et al.*, 1992). In addition, an important factor not studied until now is the exact dependence of Pgp function on [ATP]_i. It is known that cellular ATP depletion inhibits Pgp activity (for review, see Broxterman and Pinedo, 1991). However, quantitative data on ATP dependence have been reported only for isolated membrane vesicle preparations (Sarkadi *et al.*, 1992).

Another factor to be considered with regard to the detection of MDR in tumor cells is the possibility that other drug transporters, not detected by *mdr1*-specific probes, might exist, as shown in a number of non-Pgp MDR tumor cell lines (Kuiper *et al.*, 1990; Cole *et al.*, 1992; Versantvoort *et al.*, 1993).

Since functional Pgp tests for solid tumors have not been applied and validated, we measured the Pgp function in tumor-cell suspensions obtained from solid tumors, in particular human tumor xenografts, obtained by injecting cell lines with known *mdr1*/Pgp expression into nude mice. The sensitivity of a functional Pgp assay was compared with other assays which has been carried out until now for cell lines only (Herzog *et al.*, 1992). The data show that enzymatic digestion of solid tumors may yield viable tumor cells maintaining Pgp activity and allowing sensitive prediction of Pgp activity of those cells by a rapid drug-accumulation assay. This sets the stage for more accurate testing of Pgp activity and its role in clinical drug resistance.

MATERIAL AND METHODS

Cells and xenografts

A2780 and 2780^{AD} human ovarian carcinoma cells, KB3-1, KB8 and KB8-5 human epidermoid-carcinoma cells and BRO and BRO/pFR *mdr1*.6 (clone 1.1) (BRO/*mdr1*) human melanoma cells (Lincke *et al.*, 1990) were cultured in Dulbecco's MEM (Flow, Irvine, UK) supplemented with 7.5% FCS with selective drug until 2 days before use. Log-phase growing cells were harvested by trypsinization and 5×10^6 cells were injected s.c. in both flanks of 8-week-old female nude mice (athymic *nu/nu* purchased from Harlan, Zeist, The Netherlands). Groups of 2 tumor-bearing mice each were injected i.v. with 8 mg/kg DOX (Farmitalia Carlo Erba, Nivelles, Belgium). After 24 hr the tumors were excised and DOX was extracted and measured using an HPLC procedure as described (De Jong *et al.*, 1991). Per mouse 2 tumors were

³To whom correspondence and reprint requests should be sent at, Department of Medical Oncology, BR 232, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands. Fax: (31)-20-4443844.

analyzed, in duplicate. The duplicate measurements were averaged and the means of the different tumors were calculated.

Tumor dissociation procedures

Tumors (weighing 0.5 to 1.0 g) from untreated mice were excised under ether anesthesia and put into sterile Hanks's balanced salt solution. Within 30 min the tumors were cut mechanically into small pieces (several mm³). Representative parts of the tumor material were frozen directly in liquid N₂ and stored at -80°C for *mdr1* mRNA analysis. The small tumor pieces were enzymatically dissociated under continuous stirring during 30 to 60 min. at 37°C in Dulbecco's MEM, buffered with 20 mM HEPES, pH = 7.4, and supplemented with 10% FCS (medium A) + 0.14% collagenase (type I, Boehringer, Mannheim, Germany) and 0.02% DNase I (Sigma). The cell suspension was decanted over sterile nylon mesh (Zürcher Beuteltuchfabrik, Zurich, Switzerland) to remove remaining clumps and centrifuged at 250 g, washed and re-suspended in medium A. The cell suspensions were counted and checked for viability with Trypan blue. When the viability was low (usually in the case of KB8 and KB8-5 XG), dead cells were removed by a centrifugation step on Ficoll-Hypaque. Cells were frozen in liquid nitrogen for *mdr1* mRNA analysis, or kept on ice for further analysis. Daunorubicin (DNR) and vincristine (VCR) accumulation assays were started within one hour. Lactate dehydrogenase (LDH) isoenzyme analysis was done to establish the presence of mouse (stromal) cells by gel electrophoresis according to a standard procedure on a Paragon electrophoresis system from Beckmann.

Drug accumulation studies

VCR and DNR steady-state accumulation (60 min, 0.5 µM) were measured in the XG cell suspensions in direct comparison with the corresponding CL using radiolabeled drugs as published. During the incubations 0.02% DNaseI was added to prevent DNR accumulation into any non-viable cells (Versantvoort *et al.*, 1992; Broxterman *et al.*, 1988). In preliminary experiments we have checked that incubation of Pgp-expressing tumor cell lines with collagenase similar to the tumor-dissociation procedure (see above) did not significantly influence the modulator effects on DNR and VCR steady-state accumulation.

Chemosensitivity assays

Drug-sensitivity patterns were determined using a tetrazolium dye (MTT) assay (Kuiper *et al.*, 1990). Briefly, 3,000 viable (according to Trypan-blue exclusion) cells were seeded in 96-well microtiter plates in 100 µl of growth medium. After 2 hr, 100 µl of drug solution in medium was added. After 3 doubling times the medium was removed, and 50 µl MTT (Sigma) in PBS (0.4 mg/ml) were added. The plates were incubated for 3 hr at 37°C and 150 µl DMSO were added to dissolve the formed formazan. The optical density was measured on a Flow Multiscan MC plate-reader at 540 nm. All drug concentrations were tested in 4 replicate wells. Daunorubicin HCL was from Specia (Paris, France), vincristine sulphate from Sigma and bepridil from Organon (Oss, The Netherlands).

RNase protection analysis

Frozen tumor specimens were pulverized in a microdismembrator and total cellular RNA was then isolated by acid-guanidium-thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). NP40 procedure was used to isolate cytoplasmic RNA from control cell lines. RNase protection was performed as described by Baas *et al.* (1990). Briefly, 10 µg (or less as indicated) of total RNA was hybridized with a [³²P]CTP-labeled anti-sense RNA probe, specific for *mdr1* mRNA, which was obtained by transcription of a 301-nucleotide cDNA fragment (positions 3500-3801) with SP6

RNA polymerase. A probe for γ -actin was included as an internal control for determination of RNA loading. The hybridized probe was visualized after electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography (exposure to Kodak XS film overnight at -70°C).

Flow cytometry

Cells (2.10⁶) were centrifuged for 10 sec. at 1000 g in an Eppendorf centrifuge and washed 3 times in 250 µl medium A containing 1% BSA and 0.05% sodium azide (blocking buffer). The unfixed cells (10⁶) were incubated, for 1 hr at 20°C in a volume of 200 µl blocking buffer, with the anti-Pgp monoclonal antibody MRK-16 (5 µg/ml) (kindly provided by Dr. T. Tsuruo) or a non-relevant mouse IgG_{2a} (5 µg/ml) (Becton Dickinson, San Jose, CA). Samples were washed 3 times with 250 µl blocking buffer and re-suspended in 200 µl in blocking buffer supplemented with 50 µg/ml rabbit anti-mouse-IgG₁-fluorescein isothiocyanate (Dakopatts, Copenhagen, Denmark) and incubated for 30 min. at 20°C in the dark. After the pellets were washed 3 times, the cells were re-suspended in 1000 µl blocking buffer. The fluorescence of 10,000 events was logarithmically measured with a FACSTAR Plus (Becton-Dickinson, Sharon, MA) at a laser excitation of 488 nm using a 530-nm bandpass filter.

Analysis of nucleotides. Chemicals

ATP, ADP and AMP were obtained from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogen sulfate (TBAHS) was supplied by Janssen (Geel, Belgium) and HPLC-grade acetonitrile by Westburg (Leusden, The Netherlands).

Nucleotide extraction

Tumor-cell suspensions were incubated parallel to drug-accumulation assays. In order to measure nucleotide levels at the end of the accumulation period, these cells were immediately precipitated with 8% trichloroacetic acid and neutralized with triethylamine in 1,1,2-trichlorotrifluoroethane (Merck; 1/4, v/v). The extracts were stored at -80°C until analysis.

High-performance liquid chromatography

Analyses were performed on a 3-µm MicroSpher C18 column (10 cm × 4.6 mm I.D.) from Chrompack (Middelburg, The Netherlands) protected with a C18 guard column (1 cm × 3.0 mm I.D.). The injection volume was 10 µl. Separation was performed by ion-pair reversed-phase chromatography using a linear gradient. Mobile phase A consisted of 0.06 M KH₂PO₄ and 0.005 M TBAHS, adjusted to pH 6.0 with KOH. Mobile phase B was a mixture of acetonitrile and water (50/50, v/v). The column was equilibrated with 2% B before injection of the first sample. After sample injection, a linear gradient was started from 2% B at 2 min to 40% B at 12 min. The gradient returned to initial conditions at 12.2 min and the column was equilibrated for another 7.8 min. Total time between injections was 20 min. The flow rate was 1.5 ml/min and detection was performed at 254 nm. All components were quantitated by measurement of peak area. For calibration of standard solutions a millimolar extinction at 254 nm of 13.8 was used for ATP, ADP and AMP (Stocchi *et al.*, 1987).

ATP-dependence of daunorubicin accumulation

The DNR accumulation was measured as a function of ATP concentration in the KB8-5 CL. Cells were (partially) depleted of ATP by using incubations with increasing concentrations of sodium azide (Baker; Deventer, The Netherlands) or 2-deoxy-d-glucose (Sigma). After incubations of 60 min, the cells were extracted with trichloroacetic acid as described before and frozen at -80°C until HPLC analysis (27). In parallel, samples were incubated with 0.5 µM DNR with or without the Pgp-blocker bepridil (8 µM, which completely restores the reduced DNR accumulation in this cell line) and the intracel-

lular DNR accumulation was measured as described above. The medium pH was 7.4, which is not significantly different from the intracellular pH measured in this cell line (7.36 ± 0.17 ; Versantvoort *et al.*, 1992). The active efflux of DNR (v_{pump}) was calculated, by assuming that at steady-state the passive influx of uncharged DNR equals the efflux (passive + active) according to the equation:

$$k [\text{DNR}^{\text{un}}]_o = k [\text{DNR}^{\text{un}}]_i + v_{\text{pump}},$$

where k is the passive permeation coefficient, and $[\text{DNR}^{\text{un}}]_{o,i}$ is the concentration of free uncharged DNR outside or intracellularly. The DNR accumulation in the presence of 8 μM bepridil was taken as the value in the absence of Pgp pumping ($v_{\text{pump}} = 0$). Analogous to a method already described (Versantvoort *et al.*, 1994), we can now calculate the intracellular DNR concentration $[\text{DNR}]_i$. Since the medium pH was equal to pH_i , at steady-state not only the uncharged $[\text{DNR}]$ but also the total (uncharged + protonated) $[\text{DNR}]$ inside and outside the cells will be equal. Thus since also $[\text{DNR}^{\text{total}}]_o = [\text{DNR}^{\text{total}}]_i$, we can calculate v_{pump} from the above equation. The passive permeation coefficient for DNR^{un} used for the calculations was measured for the present KB8-5 cells to be $0.6 \text{ nl} \times \text{min}^{-1} \times \text{cell}^{-1}$, in accordance with published values for the KB cells (Spoelstra *et al.*, 1992).

RESULTS

In vivo MDR detection

The effect of Pgp expression on the *in vivo* tumor drug concentration was investigated by administration of DOX to the tumor-bearing mice, excision of the tumors, followed by extraction and HPLC determination of DOX and its metabolite doxorubicinol (De Jong *et al.*, 1991). The results in Table I show reduced *in vivo* accumulation of DOX in the *mdr1*-over-expressing xenografts as compared with the parent tumors. This result would be in accordance with the concept of Pgp removing drug from the tumor. Reduced *in vivo* tumor drug concentrations are in accordance with data of Rittmann-Grauer *et al.* (1992) and Horton *et al.* (1990), who showed an *in vivo* MDR phenotype of BRO/*mdr1* XG (increased *in vivo* anti-tumor effect of Vinca alkaloids by an anti-Pgp monoclonal antibody) and KB8-5 XG (increased *in vivo* vincristine accumulation by co-treatment with verapamil) respectively. Moreover, Niwa *et al.* (1992) measured reduced DOX tumor levels in KB8-5 XG. Interestingly, in our study also the accumulation of doxorubicinol, which might have been formed in the tumor cells, was lower in the Pgp-expressing tumors.

MDR detection in the KB cell lines

Since we wished to measure the function of Pgp in relation to the presence of *mdr1* mRNA or Pgp expression in the XG cells, we first established the sensitivity of the Pgp functional assay using KB3-1, KB8 and KB8-5 CL. The functional assay used is based on the determination of steady-state drug accumulation with and without Pgp reversal agent (bepridil). Table II shows that 8 μM bepridil increased DNR and VCR

accumulation significantly in KB8 CL, making the method as sensitive as *mdr1* mRNA determination (Noonan *et al.*, 1990).

MDR detection in xenografts

XG were dissociated within 30 min after removal of the tumors from the mouse. XG cell suspensions of >90% viability (Trypan blue) were obtained, except for KB XG, which had a variable viability of usually about 50% after dissociation and >80% after Ficoll separation.

Drug accumulation. The difference in cellular steady-state DNR or VCR accumulation with and without bepridil was measured and will be referred to as "Pgp activity". The results are shown in Tables II and III. Freshly dissociated BRO/*mdr1* XG cells had the same Pgp activity as BRO/*mdr1* CL. KB8-5 and KB8 XG showed a significant decrease in bepridil effect as compared with the corresponding CL. 2780^{AD} XG generally showed reduced Pgp activity (labelled "type 1"). However, 2780^{AD} XG from a minority of the mice (one example shown in Table III, labelled "type 2") had Pgp activity comparable with that of the 2780^{AD} CL.

Chemosensitivity. The MDR status of the XG cell suspensions was also measured by determining the chemosensitivity for DNR and VCR with and without 8 μM bepridil, using the MTT-assay. The results (Table IV) support the conclusion based on the short-term DNR accumulation assay (Table II), that KB8-5 and KB8 XG cells are apparently less resistant than the corresponding CL, while no difference was seen for BRO/*mdr1*. For 2780^{AD}, again 2 patterns of resistance emerged in the XG.

RNase protection. *mdr1* mRNA expression in XG was compared with that of the CL by an RNase protection assay. Representative results are shown in Figure 1. The *mdr1* mRNA expression in BRO/*mdr1* XG was comparable with that of the corresponding CL.

The 2780^{AD} XG *mdr1* mRNA expression level was dissimilar in different individual XG with no apparent differences in cell inoculation or growth rate. In most cases (designated "type 1" or XG-1) there was clearly decreased expression as compared with the 2780^{AD} cell line (estimated to be 2-fold less), while in certain cases ("type 2" or XG-2) *mdr1* expression was of similar magnitude to that in the CL. *mdr1* expression in KB8-5 XG was decreased as compared with the KB8-5 CL. Densitometric scanning of the *mdr1* and γ -actin bands revealed that the *mdr1* expression in KB8-5 XG was decreased 3-fold as compared with the KB8-5 CL.

Flow cytometry. Flow-cytometric analysis of Pgp content on the surface of XG cells, using MRK-16, showed heterogeneous distribution for all XG as compared with the respective cell lines, except for 2780^{AD} ("type 2") (see Fig. 2). The data for Pgp expression in the KB8-5 XG were inconclusive for that reason. 2780^{AD} and BRO/*mdr1* Pgp expression levels were in accordance with the *mdr1* mRNA data.

For the KB8-5, an experiment was done to determine whether the decreased Pgp expression and function in XG cells might be due in part to contamination with mouse stroma cells. However, since we found no apparent mouse LDH-V band in fresh KB8-5 XG cells and because the percentage of keratin-expressing cells was around 100% (except for a small rise in the percentage of keratin-negative cells upon subsequent *in vitro* culture, probably corresponding to transient outgrowth of mouse fibroblasts), we excluded that the decrease in *mdr1* mRNA and Pgp activity in KB XG could be due to significant mouse-cell contamination.

Extended cell culture of XG cells

To obtain additional information on the relation between Pgp content and Pgp function in the different dissociated XG, the freshly obtained cell suspensions were cultured *in vitro* and the resulting "cell line" (XG-CL), after several weeks of

TABLE I - DOXORUBICIN AND DOXORUBICINOL LEVELS IN XENOGRAPHS

Tumor type	Doxorubicin (nmol/g $\pm$ SD)	Doxorubicinol (nmol/g $\pm$ SD)
A2780	5.4 ± 0.7 (n = 4)	0.07 ± 0.01 (n = 4)
2780 ^{AD}	1.3 ± 0.3 (n = 2)	0.01 (n = 1)
KB3.1	8.8 ± 1.6 (n = 4)	0.15 ± 0.03 (n = 4)
KB8.5	5.1 ± 0.8 (n = 4)	0.06 ± 0.01 (n = 4)
BRO	4.8 ± 0.4 (n = 4)	0.02 ± 0.003 (n = 4)
BRO/ <i>mdr1</i>	2.2 ± 0.5 (n = 4)	n.d.

Data are mean $\pm$ SD; n = number of tumors analyzed; n.d., not detectable.

TABLE II – EFFECT OF 8 μ M BEPRIDIL ON THE ACCUMULATION OF DAUNORUBICIN AND VINCRISTINE IN CELL LINES (CL) AND XENOGRAPHS (XG)

	Cell line		XG cell suspension		
	DNR	VCR	After dissociation		After culture DNR
			DNR	VCR	
BRO	98	131	103	139	
BRO/mdr1	187 $\pm$ 32	230 $\pm$ 32	196 $\pm$ 21	240 $\pm$ 18	169 $\pm$ 24
KB3-1	98 $\pm$ 6	139 $\pm$ 6	99	115	
KB8	129 $\pm$ 14	221 $\pm$ 38	88 $\pm$ 4	101 $\pm$ 8	123
KB8-5	231 $\pm$ 65	458 $\pm$ 254	131 $\pm$ 19	159 $\pm$ 5	248 $\pm$ 24

Data are expressed as percentage of drug accumulation in the same cells without modifier (=100%). Data are mean $\pm$ SD of at least 3 experiments; no SD means that only one experiment was performed.

TABLE III – EFFECT OF 8 AND 64 μ M BEPRIDIL ON THE ACCUMULATION OF DAUNORUBICIN IN 2780^{AD} CELLS

2780 ^{AD}	Control	+8 μ M bepridil	+64 μ M bepridil
Cell line	5 $\pm$ 6	52 $\pm$ 13 (1040%)	128 $\pm$ 21 (2560%)
Xenograft, type 1 after dissociation	41 $\pm$ 11	76 $\pm$ 11 (185%)	86 $\pm$ 8 (210%)
after culture	49 $\pm$ 17	106 $\pm$ 36 (216%)	129 $\pm$ 6 (263%)
Xenograft, type 2 after dissociation	8	39 (488%)	
after culture	1	28 (2800%)	94 (9400%)

Data are expressed in pmol/10⁶ cells $\pm$ SD of 3 to 5 experiments.

TABLE IV – EFFECT OF 8 μ M BEPRIDIL ON THE CHEMOSENSITIVITY OF DAUNORUBICIN AND VINCRISTINE OF CELL LINES (CL) AND XENOGRAPHS (XG)

	Daunorubicin		Vincristine	
	DMF	RF	DMF	RF
BRO				
CL	1.5 $\pm$ 0.5		1.5 $\pm$ 0.3	
BRO/mdr1				
CL	7 $\pm$ 2	18 $\pm$ 6	39 $\pm$ 15	57 $\pm$ 1
XG	6 $\pm$ 3	17 $\pm$ 3	27 $\pm$ 12	52 $\pm$ 19
KB3-1				
CL	1.0 $\pm$ 0.3		2.1 $\pm$ 0.2	
XG	1.2 $\pm$ 0.2		1.5 $\pm$ 0.1	
KB8				
CL	1.0 $\pm$ 0.0	3.6 $\pm$ 0.4	5.3 $\pm$ 1.8	8.3 $\pm$ 4.4
XG	1.1 $\pm$ 0.2	1.2 $\pm$ 0.3	2.8 $\pm$ 0.4	2.6 $\pm$ 0.8
KB8-5				
CL	2.4 $\pm$ 0.7	3.4 $\pm$ 1.3	24.2 $\pm$ 8.7	88 $\pm$ 20
XG	1.2 $\pm$ 0.5	0.6 $\pm$ 0.6	7.9 $\pm$ 2.1	21 $\pm$ 12
2780 ^{AD}				
CL	24.4 $\pm$ 8.8	190 $\pm$ 40	48.6 $\pm$ 18.2	1025 $\pm$ 300
XG, type 1	2.7	8	10.2	30
XG, type 2	16.4 $\pm$ 1.6	108 $\pm$ 35	32.4 $\pm$ 2.3	925 $\pm$ 35

Data are means $\pm$ SD of at least 2 experiments. DMF (dose-modifying factor) is IC₅₀ without bepridil, divided by IC₅₀ with bepridil. RF (resistance factor) is IC₅₀ MDR cell line divided by IC₅₀ parent cell line.

growth, was analyzed again for mdr1 mRNA, Pgp and Pgp function. Cells from the XG readily attached to the culture flasks in the case of BRO/mdr1 and 2780^{AD}, but only for a small percentage in case of KB8-5. The MDR phenotype (RNA, not shown; Pgp, Fig. 2; and Pgp activity, Tables II, III) of BRO/mdr1 and KB8-5 XG-CL (grown without *in vitro* drug-selection pressure) was similar to the respective CL. Thus the Pgp function in KB8-5 XG-CL was completely recovered. However, the 2780^{AD} XG-CL (type 1), grown without DOX, had an MDR phenotype similar to the XG, but became more

FIGURE 1 – mdr1 mRNA levels in cell lines (CL), xenograft tissue (XG solid) and cell suspensions from XG (XG susp). 2780^{AD} XG appeared to have different expression levels (XG-I and XG-II, see text). RNase protection assay on 10 μ g (unless otherwise indicated) cytoplasmic RNA, using mdr1 and γ -actin probes. The position of the protected fragments is indicated. Probes for mdr1 and actin and tRNA control are shown in the lanes designated.

resistant when grown with 2 μ M DOX (see Fig. 2b, “XG cultured *in vitro* + DOX”). In the latter case, however, many XG cells did not survive, indicating selection of a sub-population of more resistant cells.

Analysis of nucleotides

Since an ATP-dependent drug pump requires the presence of ATP in order to function, we measured the levels of ATP, ADP and AMP in the XG and CL. Moreover, there is no information available concerning the level of [ATP]_i depletion at which Pgp activity will become impaired in intact cells. Since solid tumors generally have considerably lower ATP levels than *in vitro* cultured cell lines (Kuhnle *et al.*, 1992; Vaupel *et al.*, 1989), we approached this question by reducing KB8-5 CL to different ATP levels by applying a range of concentrations of sodium azide and deoxyglucose. Then again the effect of bepridil on DNR accumulation in these cells was measured to probe Pgp activity. From this measurement, we could calculate the v_{pump} for Pgp in KB8-5 cells at a fixed [DNR]_i for cells with different ATP levels (Fig. 3). A half-maximal inhibition of active DNR transport was found at approximately 1.5 to 3.0 nmol/10⁶ cells, (or at a calculated [ATP]_i = 1 to 2 mM, assuming homogeneous distribution of ATP over the whole

FIGURE 2 – Pgp expression in cell lines, xenograft cell suspension (XG) and XG cultured *in vitro*. Flow cytometry with anti-Pgp antibody MRK-16 (solid lines) or irrelevant immunoglobulin (dotted lines).

cell volume; see Fig. 3). Table V shows that, although the ATP levels of the XG cell suspensions was lower than in the corresponding cell line, they are around (KB8-5) or above 3.0 nmol/ 10^6 cells.

DISCUSSION

Levels of *mdr1* mRNA expression in certain human tumors, such as renal-cell cancer and colon cancer, are in the same order of magnitude as the levels found in KB8 or KB8-5 MDR cell lines (Noonan *et al.*, 1990), suggesting that Pgp might play a role in resistance to chemotherapy in these tumor types. In order to translate the knowledge relating to Pgp-mediated resistance in cell lines to a clinical situation, it is necessary to know the impact of a certain level of *mdr1*/Pgp expression *in vivo* on the intracellular drug steady-state concentration or drug cytotoxicity. With regard to clinical usefulness of an MDR parameter, it would be desirable to assess the effect of a resistance modifier on net drug uptake or cytotoxicity, to decide on a rationale for co-treatment with a resistance modifier. Since cells from clinical tumor samples are difficult to grow *in vitro*, a short-term drug-uptake assay might be more within reach. Drug (mainly anthracycline) uptake in fresh human tumor cells has been explored in only a few cases, exclusively in hematological malignancies (Nooter *et al.*, 1990; Schuurhuis *et al.*, 1995). No reports on screening of solid tumors for Pgp activity by drug accumulation assays have appeared until now. Consequently, there are no experimental data showing the impact of Pgp expression on drug transport in solid tumors.

FIGURE 3 – ATP-dependence of Pgp activity in intact KB8-5 cells. The DNR efflux rate (v_{pump}) was determined from the DNR accumulation data as described in Material and Methods, normalized to $[\text{DNR}]_i$ of 0.5 μM . The curve was drawn using a V_{max} of 60 pmol/ 10^6 cells/min, a K_m of 1 mM (Shapiro and Ling, 1994) and a Hill coefficient (Hill, 1985). $n = 2$ (data not shown). Symbols are: +, glucose-rich medium; $\diamond$, medium without glucose; $\circ$, medium without glucose, with sodium azide; $\triangle$, medium without glucose, with azide and deoxyglucose.

TABLE V - LEVELS OF ATP, ADP AND AMP IN TUMOR XENOGRAPHS (XG) AND CELL LINES (CL)

	ATP	ADP	AMP	ATP + ADP + AMP	n
BRO/mdr1					
XG	4,635 ± 432	1,250 ± 372	393 ± 66	6,278	3
CL	7,026 ± 732	1,259 ± 531	17 ± 24	8,302	2
KB8-5					
XG	2,883 ± 251	1,113 ± 431	615 ± 271	4,611	3
CL	7,414 ± 632	1,481 ± 632	699 ± 366	9,594	8
2780 ^{AD}					
XG	3,331 ± 380	492 ± 88	424 ± 6	4,247	2
CL	4,197 ± 534	1,100 ± 120	0	5,297	3

Data are expressed in pmol/10⁶ cells (mean ± SD of 2 to 8 experiments).

In this report we address essential questions such as the feasibility, sensitivity and specificity of a functional test for the presence of active drug transporters in a solid tumor by using human tumor XG with different levels of mdr1 expression as a model. Although the cellular heterogeneity of human solid tumors, which represents a further complication, remains unaddressed in this study, our method of dissociating the XG into cell suspensions can in principle be used to obtain single cells from human solid tumors.

In general, our results show that it is possible to predict an MDR phenotype in freshly isolated solid tumor cells by a DNR or VCR accumulation assay. The results of this assay were in accordance with the chemosensitivity assay after continuous 72-hr exposure to the drugs DNR and VCR. This implies that Pgp present in metabolically active tumor cells will not be damaged during the period of removal of the tumor and dissection/enzymatic digestion. Thus, the difference in Pgp activity (modulator effect) as seen in individual 2780^{AD} XG ("type 1" vs. "type 2") appeared to reflect the difference in *in vivo* mdr1/Pgp expression, which for unknown reasons varied in this model. Also the decreased Pgp function in KB8-5 XG cells compared with the KB8-5 CL could be explained by decreased *in vivo* mdr1 mRNA expression in this cell line. In the BRO/mdr1 model, no decrease in Pgp function or in expression was noted when compared with the CL. Moreover, from these data also the energetic state of the dissociated XG cells appeared to be sufficient to support the function of the Pgp pump.

To obtain more quantitative data on the requirement of a plasma-membrane drug transporter such as Pgp for ATP in intact cells, we varied the ATP content in KB8-5 cells. This experiment showed that half-maximal inhibition of DNR transport was obtained at 1.5 to 3.0 nmol ATP/10⁶ cells or at an [ATP]_i of 1 to 2 mM, assuming a homogeneous ATP distribution over the whole cell volume. This value is somewhat higher than some published values for the apparent K_m for ATP of Pgp ATPase activity in isolated membranes, which are in the order of 0.5 to 1.4 mM (Sarkadi *et al.*, 1992; Al-Shawi and Senior, 1993; Broxterman and Versantvoort, 1995). An apparent K_m of 0.94 mM was published recently for highly purified Pgp (Shapiro and Ling, 1994). In any case, our data mean that inhibition of Pgp activity may occur in intact tumor cells at ATP levels below 2 mM (see Fig. 3). These considerations are of relevance, since ATP levels in hamster melano-

mas were found to be in a range of 0.1 to 2 mM throughout the tumor (Kuhnle *et al.*, 1992), with values below 0.5 mM occurring in apparently viable areas, while average ATP concentrations of about 1 mM have been reported in human breast-cancer xenografts (Vaupel *et al.*, 1989).

It is not known whether tumor regions with low levels of ATP would yield viable cells after a dissociation procedure. However, the KB8-5 XG we used in this study had a less vascularized appearance and a much higher percentage of non-viable cells than the other XG. Nevertheless (a relatively low number of) cells with a reasonably high ATP level were recovered. Since the decreased Pgp function in KB8-5 XG could be explained to a large extent by the decrease of mdr1 mRNA expression *in vivo* (which also occurred in 2780^{AD} type 1 XG) as compared with the CL, the contribution of a shortage of ATP available for hydrolysis by the Pgp ATPase could have been only small in this case. Other factors that theoretically may contribute to altered Pgp activity, such as altered translation, altered Pgp phosphorylation state or altered passive membrane permeability, all seem to be of minor importance.

The KB8-5 XG might be more similar to a human solid tumor than the other models presented, because it has low mdr1 expression and a high percentage of necrotic cells. Also, the quantitative analysis of Pgp expression in KB8-5 XG cells was more difficult, since an increase in heterogeneity of tumor cells, many dead cells and some stromal cells all impair the flow-cytometric analysis, which was at the limit of detection in this case. Despite these problems, the "*in vivo* MDR phenotype" as inferred from the DOX tumor uptake could be reasonably well predicted from the *in vitro* drug accumulation assay.

Other drug transporters, such as MRP can be analyzed in the same way when a specific modulator for such a transporter is known. One candidate-type of modulator is the isoflavonoid genistein (Versantvoort *et al.*, 1993). Applying our approach to human tumors will lead to more insight into the quantitative role of drug transporters in clinical multidrug resistance.

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