



## COMPETITIVE INHIBITION BY GENISTEIN AND ATP DEPENDENCE OF DAUNORUBICIN TRANSPORT IN INTACT *MRP* OVEREXPRESSING HUMAN SMALL CELL LUNG CANCER CELLS

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**Abstract**—In several multidrug resistant tumor cell lines without overexpression of P-glycoprotein (non-Pgp MDR), a decreased accumulation of drugs has been shown to contribute to resistance. We have recently reported that daunorubicin (DNR) accumulation was decreased in the multidrug resistance-associated protein overexpressing GLC4/ADR non-Pgp MDR small cell lung cancer cell line due to an enhanced energy-dependent efflux which could be inhibited by the isoflavonoid genistein. The purpose of this work was 2-fold: (i) to investigate the mechanism by which genistein inhibits the DNR efflux in the GLC4/ADR cells; and (ii) to characterize the dependence of DNR transport on ATP concentration in intact GLC4/ADR cells. The active transport of DNR in GLC4/ADR cells appeared to be a saturable process with an apparent  $K_m$  of DNR of  $1.4 \pm 0.4 \mu\text{M}$ . Genistein increased the apparent  $K_m$  value of DNR, suggesting that this agent is a competitive inhibitor of DNR transport. These data provide additional evidence that energy-dependent DNR transport in GLC4/ADR cells is a protein-mediated process. In addition, genistein decreased cellular ATP concentration in a dose-dependent manner in sensitive as well as in resistant cells. Marked inhibition of DNR transport activity in intact GLC4/ADR cells was found when cellular ATP concentration was decreased below 2 mM by sodium azide or 2-deoxy-D-glucose. Thus, since DNR transport in intact GLC4/ADR is already inhibited at modest cellular ATP depletion, a limitation in ATP supply might open ways to make MDR cells more susceptible to drug toxicity.

**Key words:** multidrug resistance; cellular daunorubicin accumulation; ATP; genistein; *MRP*; (non) P-glycoprotein

Resistance of tumor cells to cytostatic agents is one of the problems that limit the success of chemotherapeutic treatment of cancer. Overexpression of the *MDR1* gene, which encodes for the plasma membrane protein Pgp<sup>†</sup>, has been demonstrated *in vitro* to cause resistance to multiple drugs (MDR). Pgp belongs to the superfamily of ATP-binding transporters and reduces intracellular drug concentrations by expelling drugs from the cell in an ATP-dependent manner [1, 2].

Recently, a decreased accumulation of drugs has been demonstrated in cell lines that are selected *in vitro* for resistance but do not show overexpression of Pgp [3–8]. In several of these so-called non-Pgp MDR cells drug accumulation was nevertheless shown to be decreased due to an enhanced drug efflux [4, 6, 7, 9]. This enhanced drug efflux was

blocked when cellular ATP synthesis was completely inhibited by metabolic inhibitors. Moreover, we have shown that outward DNR transport occurs against a concentration gradient [10], indicating the presence of active drug transport in non-Pgp MDR cells.

Recently, Cole *et al.* [11] have cloned a gene, called MDR-associated protein (*MRP*), which is likely to encode a drug transporter protein in many non-Pgp MDR cells [11]. Transfection of the *MRP* gene has been shown to confer the MDR phenotype to drug-sensitive HeLa cells [12]. These *MRP*-transfectants also overexpress a *M*, 190,000 integral membrane protein which is known to be overexpressed in a number of other non-Pgp MDR cell lines (HL60/ADR, HT1080/DR4, COR-L23/R, MOR/R and GLC4/ADR) [13–16]. These cell lines have in common a decreased drug accumulation.

Although non-Pgp MDR is phenotypically very similar to Pgp MDR, resistance modulators of Pgp MDR such as verapamil and cyclosporin A are usually less effective in non-Pgp MDR cells [8, 17]. Recently, we have shown that decreased DNR accumulation in several non-Pgp MDR cell lines could be reversed by the isoflavonoid genistein, a tyrosine kinase inhibitor [23], which did not cause an increase in DNR accumulation in Pgp MDR cells [18]. Because of this differential effect of genistein on DNR accumulation in Pgp and non-Pgp MDR

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† Abbreviations: MDR, multidrug resistance (resistant); Pgp, P-glycoprotein; *MRP*, multidrug resistance-associated protein; DNR, daunorubicin; NaN<sub>3</sub>, sodium azide; DG, 2-deoxy-D-glucose; [DNR<sup>un</sup>] and [DNR], concentration of the uncharged form or total (uncharged and protonated) daunorubicin, respectively; [DNR]<sub>i</sub>/[DNR]<sub>o</sub>, intra-/extracellular daunorubicin concentration; pH<sub>i</sub>, intracellular pH; pH<sub>o</sub>, extracellular pH;  $v_{\text{pump}}$ , active efflux rate;  $v_i/v_o$ , passive influx/efflux rate.

cells, genistein might be used to probe non-Pgp mediated MDR by using a DNR accumulation assay. The precise mechanism of action for genistein is not known, but our data make it unlikely that a tyrosine kinase mediated step was involved [18].

In an attempt to gain an insight into the mechanism of action of genistein on DNR accumulation in non-Pgp MDR cells, we now report a pharmacological characterization of the DNR transporter present in the human small cell lung carcinoma cell line, GLC4/ADR. GLC4/ADR cells have been demonstrated to overexpress the *MRP* gene [16], which is therefore likely to be the gene which encodes the drug transporter present in GLC4/ADR cells. First, we show that DNR transport in non-Pgp MDR GLC4/ADR cells is saturable. Then, evidence for competitive inhibition of this DNR transport by genistein is provided. In addition, the dependence of DNR transport on ATP concentrations in intact cells is determined. Until now only the complete inhibition of drug transporter activity upon complete ATP depletion had been reported. Moreover, measurements in intact cells are of importance because these data would predict the *in vivo* ATP concentrations needed for effective DNR transport in contrast to experiments using (partial) purified transporter protein(s), such as for Pgp, which establish *in vitro*  $K_m$  values for the transporter ATPase. Our data show reduced effectiveness of the DNR transporter in GLC4/ADR cells already at cellular ATP concentrations of <2 mM.

#### MATERIALS AND METHODS

**Materials.** Daunorubicin hydrochloride and genistein were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [ $G-^3H$ ]DNR hydrochloride (sp. act. 1.6 Ci/mmol) was from DuPont de Nemours (Germany). ATP and ADP were obtained from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogen sulfate (TBAHS) was supplied by Janssen Chimica (Geel, Belgium).

**Cells.** The human small cell lung carcinoma cell line GLC4 and its doxorubicin-selected non-Pgp MDR subline GLC4/ADR [8] were cultured in RPMI medium (Flow Labs., Irvine, U.K.) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, U.K.). The resistant cell lines were cultured in the presence of 1.2  $\mu$ M doxorubicin until 2–14 days before experiments.

**Cellular DNR accumulation.** Cellular steady-state accumulation of [ $^3H$ ]DNR was measured as described previously [9]. Briefly, cells were incubated at 37° in growth medium without sodium bicarbonate supplemented with 10% fetal calf serum (pH 7.35). DNaseI (0.02%) was included to prevent DNR accumulation in any non-viable cells. DNR uptake was initiated by addition of the radiolabeled drug and after 60 min the cells were washed twice with ice-cold PBS.

**ATP measurements.** In order to measure ATP dependence of DNR transport, cells ( $1-2 \times 10^6$  cells/mL) were incubated parallel to DNR accumulation. Cellular ATP was depleted by incubation of the cells in medium without glucose and with addition of  $NaN_3$  and/or DG. After cooling on ice, the cells

were pelleted and ATP and ADP were determined in cellular trichloroacetic acid extracts by HPLC analysis [19]. Separation of the ribonucleosides was performed by ion-pair reversed-phase chromatography using a linear gradient (2–40% phase B) on a 3- $\mu$ m MicroSpher C18 column (10 cm  $\times$  4.6 mm i.d.) from Chrompack (Middelburg, The Netherlands) with a C18 precolumn (1 cm  $\times$  3.0 mm i.d.). Phase A consisted of 60 mM  $KH_2PO_4$  and 5 mM TBAHS (pH 6.0) and phase B was a 50% acetonitrile solution in water (v/v). Detection was performed at 254 nm and the components were quantitated by measurement of peak area. Intracellular ATP concentration ( $[ATP]_i$ ) was calculated based on cell volumes as estimated from their diameters ( $13.2 \pm 0.2 \mu$ m for GLC4 and GLC4/ADR cells).

**Kinetic modelling.** Based on our previously reported kinetic data on DNR transport in GLC4/ADR cells [9], DNR transport in these cells could be described by the “leak and pump” model [20]: a passive diffusion of the uncharged form ( $v_i$  and  $v_o$ ) and the active efflux of DNR ( $v_{pump}$ ) (Fig. 1). The influx rate ( $v_i$ ) of DNR increased proportionally with DNR concentration in the medium ( $0.5 \leq [DNR]_o \leq 10 \mu$ M data not shown). Thus, at least in this concentration range, the influx of DNR followed simple diffusion kinetics. We previously measured, under the same conditions as used here, a passive-permeation coefficient ( $k$ ) of 21  $\mu$ L/ $10^6$  cells/min in GLC4/ADR cells [9]. At steady-state, the passive influx of DNR equals the efflux (passive and active) according to eqn 1:

$$k[DNR^{un}]_o = k[DNR^{un}]_i + v_{pump} \quad (1)$$

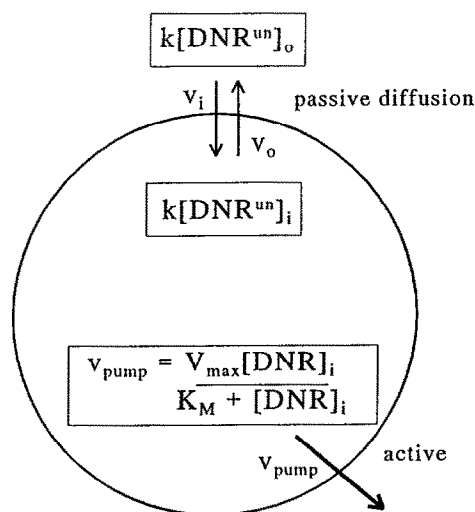


Fig. 1. Schematic representation of DNR transport in GLC4/ADR cells. The transport of DNR is described by passive diffusion of the uncharged form of DNR ( $v_i$  and  $v_o$ ) and an energy depending efflux ( $v_{pump}$ ):  $k$ , passive permeation coefficient;  $[DNR^{un}]_i/[DNR^{un}]_o$ , intra-/extracellular concentration of the uncharged form of DNR;  $[DNR]_i/[DNR]_o$ , intra-, extracellular total DNR concentration;  $V_{max}$ , maximal pump rate for DNR;  $K_m$ , Michaelis-Menten constant.

In sensitive cells ( $v_{\text{pump}} = 0$ ) at steady-state  $[\text{DNR}]_{\text{i}}$  is equal to  $[\text{DNR}]_{\text{o}}$ . Since DNR accumulation was measured at a medium pH of 7.35, which is equal to  $\text{pH}_i$  [9], at steady-state both the uncharged DNR concentration as well as total (uncharged and protonated form) DNR concentration inside and outside the cell have to be equal: ( $[\text{DNR}]_{\text{o}} = [\text{DNR}]_{\text{i}}$  and  $[\text{DNR}]_{\text{i}} = [\text{DNR}]_{\text{o}}$ ). We demonstrated previously that permeabilization of the plasma membrane with digitonin had no effect on DNR accumulation in parental GLC4 cells, proving that  $[\text{DNR}]_{\text{i}}$  was indeed  $[\text{DNR}]_{\text{o}}$  in these cells [10]. On the other hand DNR accumulation in resistant GLC4/ADR cells was increased in response to digitonin, indicating that cellular DNR accumulation was reduced against a concentration gradient. The same amount of DNR was bound to sensitive and resistant cells once the plasma membrane was permeabilized [10]. These resistant and sensitive cells thus have the same DNR binding capacity. Since DNR accumulation (predominantly bound to DNA) is linear with  $[\text{DNR}]_{\text{o}}$  in sensitive cells, the  $[\text{DNR}]_{\text{i}}$  in resistant GLC4/ADR cells could be calculated by measuring the accumulation defect (eqn 2)

$$[\text{DNR}]_{\text{i}} = \frac{(\text{DNR acc.})_{v_{\text{pump}}=0}}{(\text{max. DNR acc.})_{v_{\text{pump}}=0}} [\text{DNR}]_{\text{o}} \quad (2)$$

For (maximal DNR accumulation) $_{v_{\text{pump}}=0}$  we used DNR accumulation in the GLC4 cells, given that both cells have the same DNR binding capacity (see above). Once  $[\text{DNR}]_{\text{i}}$  is known,  $v_{\text{pump}}$  can then be calculated according to eqn 1.

For kinetic studies under cellular ATP depleting conditions it was assumed that DNR binding capacity was not affected. However, cellular ATP depletion results in an increase in DNR accumulation in sensitive cells, probably due to decreased  $\text{pH}_i$  [9, 20, 21]. Since this  $\text{pH}_i$  decrease as well as DNR accumulation upon energy depletion were the same in sensitive and resistant GLC4 cells [9], we corrected the DNR accumulation in the GLC4/ADR cells for the effects of  $\text{NaN}_3$  and DG on DNR accumulation measured in the sensitive cells.

In order to examine the dependence of active DNR transport on  $[\text{ATP}]_{\text{i}}$  by the above described kinetic analysis, DNR accumulation and  $[\text{ATP}]_{\text{i}}$  had to be constant at the measured time point (60 min). When incubation time was prolonged to 90 min, neither  $[\text{ATP}]_{\text{i}}$  nor DNR accumulation data changed by more than 15%, indicating that DNR accumulation and  $[\text{ATP}]_{\text{i}}$  were indeed near steady-state at 60 min.

## RESULTS

### Saturation of DNR transport in GLC4/ADR cells

DNR accumulation was measured within a range of DNR concentrations in order to determine saturation of active DNR transport in GLC4/ADR cells. DNR accumulation divided by  $[\text{DNR}]_{\text{o}}$  (distribution volume) is presented as a function of  $[\text{DNR}]_{\text{o}}$  in Fig. 2A. In parental GLC4 cells DNR accumulation appeared to be linear with  $[\text{DNR}]_{\text{o}}$ , as illustrated by the horizontal curve. This is consistent with DNR

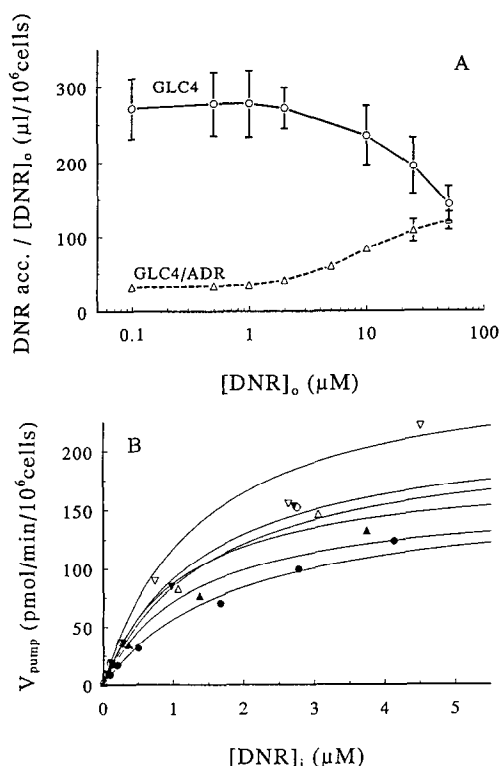


Fig. 2. Saturation of DNR transport in GLC4/ADR cells. (A) Steady-state accumulation at various  $[\text{DNR}]_{\text{o}}$  was measured in parental GLC4 ( $\circ$ ) and resistant GLC4/ADR ( $\triangle$ ) cells. Data are means  $\pm$  SD of three to six experiments; no error bar means SD was smaller than the symbol. (B) ATP-dependent drug efflux rate of the data shown in A (six separate experiments).

transport across the plasma membrane by passive diffusion. The relative decrease of DNR accumulation at  $[\text{DNR}]_{\text{o}} > 5 \mu\text{M}$  is probably caused by saturation of DNR binding to DNA (decrease in distribution volume). In resistant GLC4/ADR cells DNR accumulation appeared to be linear with  $[\text{DNR}]_{\text{o}}$  up to  $[\text{DNR}]_{\text{o}}$  of 2  $\mu\text{M}$ . However at  $[\text{DNR}]_{\text{o}} > 2 \mu\text{M}$ , a relative increase of DNR accumulation was observed with increasing DNR concentrations, which most likely can be explained by a saturation of active DNR transport.

From these DNR accumulation studies we calculated intracellular DNR concentration ( $[\text{DNR}]_{\text{i}}$ ) and the active DNR efflux rate ( $v_{\text{pump}}$ , Fig. 1) in GLC4/ADR cells as outlined in Materials and Methods. The binding of DNR to DNA was shown to be linear with  $[\text{DNR}]_{\text{i}}$  up to 5  $\mu\text{M}$  in GLC4 cells (Fig. 2A). Therefore, only data with  $[\text{DNR}]_{\text{i}} < 5 \mu\text{M}$  were taken into account in calculating  $v_{\text{pump}}$  (Fig. 2B). The  $v_{\text{pump}}$  was saturable and followed first-order Michaelis–Menten kinetics. An apparent  $K_m$  of  $1.4 \pm 0.4 \mu\text{M}$  and  $V_{\text{max}}$  of  $203 \pm 44 \text{ pmol DNR}/10^6 \text{ cells}/\text{min}$  were calculated from six independent experiments by least-squares analysis of Eadie–Hofstee plots (Fig. 3B). A similar  $K_m$  value was

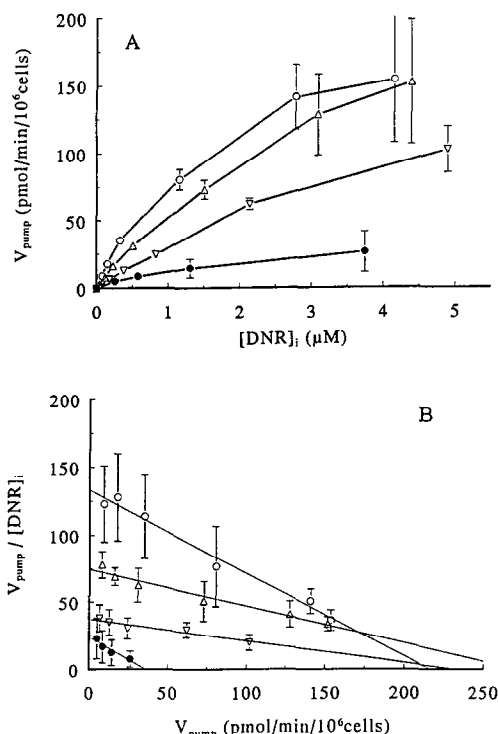


Fig. 3. Inhibition of DNR transport by genistein and  $\text{NaH}_2\text{PO}_4$ . (A) Inhibition of DNR transport rate in GLC4/ADR cells by 50  $\mu\text{M}$  genistein ( $\Delta$ ), 200  $\mu\text{M}$  ( $\blacktriangledown$ ),  $\text{NaH}_2\text{PO}_4$  without glucose in the medium ( $\text{ATP} = 1.2 \pm 0.6 \text{ mM}$ ) ( $\bullet$ ) or control ( $\circ$ ) as function of  $[\text{DNR}]_i$ . (B) Eadie-Hofstee plots of data shown in A. Each data point represents mean  $\pm$  SD of three to six independent experiments.

found for DNR transport in intact Pgp MDR cells [21].

#### Competition for DNR transport in GLC4/ADR cells by genistein

These experiments were then repeated in the presence of genistein in order to determine whether inhibition of the enhanced DNR efflux by genistein [18] was competitive or non-competitive with DNR.

Since inhibition of transporters by ATP depletion is usually not competitive with the transported compound, in this experiment we compared the effect of ATP depletion with the effect of genistein (Fig. 3A).

Linear transformation of the data (Fig. 3A) in the presence or absence of 50 and 200  $\mu\text{M}$  genistein or in partially ATP depleted cells ( $\text{ATP} = 1.2 \pm 0.6 \text{ mM}$ ), resulted in the lines illustrated in Fig. 3B. Cellular ATP depletion, as expected, displayed non-competitive inhibition kinetics; i.e. a decrease in  $V_{\text{max}}$  without affecting the apparent  $K_m$  value (Table 1). Genistein, in contrast, increased the apparent  $K_m$  value in a dose-dependent manner without affecting the  $V_{\text{max}}$  (Table 1). Thus, genistein appeared to be a competitive inhibitor of active DNR transport in GLC4/ADR cells.

#### Dependence of DNR transport on $[\text{ATP}]_i$

The enhanced DNR efflux in GLC4/ADR cells was completely blocked in medium without glucose but with 10 mM  $\text{NaN}_3$  and 6 mM DG, which leads to cellular ATP depletion of >95% (Table 2 [9]). In order to study ATP dependence in more detail, intermediate cellular  $[\text{ATP}]_i$  were obtained by using different concentrations of DG and  $\text{NaN}_3$  with or without glucose in the medium (Table 2). In Fig. 4A DNR accumulation as a function of  $[\text{ATP}]_i$  is shown. In ATP depleted GLC4 cells DNR accumulation was increased to a small extent (110–130%) probably due to changes in  $\text{pH}_i$  [9]. The accumulation of DNR in GLC4/ADR cells was reduced  $\approx 7$ -fold compared to GLC4 cells under standard conditions ( $> 3 \text{ mM}$   $[\text{ATP}]_i$ ). In GLC4/ADR cells DNR accumulation increased progressively when  $[\text{ATP}]_i$  dropped below 2 mM, reaching the level of parental cells at  $[\text{ATP}]_i$  less than 0.2 mM.

In order to determine the apparent affinity of the DNR transporter for ATP in intact GLC4/ADR cells,  $v_{\text{pump}}$  was calculated as described in Materials and Methods. Since the accumulation studies were performed at a constant (0.5  $\mu\text{M}$ )  $[\text{DNR}]_o$ , the calculated  $[\text{DNR}]_i$  in GLC4/ADR cells varied between 0.08 and 0.5  $\mu\text{M}$  under different energy depleting conditions. To determine the affinity for ATP of DNR transport in GLC4/ADR cells, we normalized  $v_{\text{pump}}$  values to a  $[\text{DNR}]_i$  of 0.5  $\mu\text{M}$ . We did so because  $v_{\text{pump}}$  is approximately linear with  $[\text{DNR}]_i$  in this concentration range and the effects of ATP

Table 1. Kinetic parameters of DNR transport in GLC4/ADR cells

Conditions	$K_m$ ( $\mu\text{M}$ )*	$V_{\text{max}}$ (pmol/10 <sup>6</sup> cells/min)*
Control (6)	$1.4 \pm 0.4$	$203 \pm 44$
+50 $\mu\text{M}$ genistein (4)	$3.6 \pm 0.5^\dagger$	$267 \pm 72$
+200 $\mu\text{M}$ genistein (6)	$5.7 \pm 2.2^\dagger$	$201 \pm 59$
–glucose + 10 mM $\text{NaN}_3$ (3)	$1.4 \pm 0.3$	$35 \pm 22^\dagger$

\*  $K_m$  and  $V_{\text{max}}$  values were calculated from Eadie-Hofstee plots; data are means  $\pm$  SD of least-squares analysis of three to six independent experiments (number of experiments in parentheses).

† Significantly different,  $P < 0.01$  compared to control according to Student's *t*-test.

Table 2. Intracellular ATP in presence of metabolic inhibitors

	[ATP] <sub>i</sub> in mM*	
	GLC4	GLC4/ADR
Control	4.4 ± 1.4 (6)	4.7 ± 1.1 (11)
+10 mM NaN <sub>3</sub>	3.3 ± 2.0 (4)	4.1 ± 1.1 (9)
+6 mM DG	3.9, 4.6	2.7 ± 0.6 (5)
-glu + 10 mM NaN <sub>3</sub>	1.3 ± 1.1 (3)	1.2 ± 0.6 (8)
-glu + 6 mM DG	1.5 ± 0.7 (4)	1.2 ± 0.3 (7)
-glu + 10 mM NaN <sub>3</sub> + 0.6 mM DG	n.d.	0.48 ± 0.30 (3)
-glu + 10 mM NaN <sub>3</sub> + 1.3 mM DG	n.d.	0.27 ± 0.13 (3)
-glu + 10 mM NaN <sub>3</sub> + 6 mM DG	0.17 ± 0.14 (3)	0.10 ± 0.06 (5)

\* Data are means ± SD of 2–11 experiments, number of experiments in parenthesis.

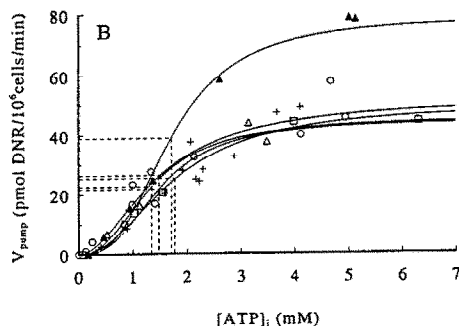
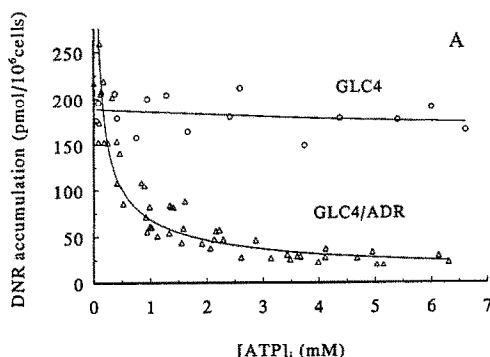


Fig. 4. ATP concentration dependence. (A) ATP-dependent DNR accumulation was measured as a function of ATP concentration in GLC4 (○) and GLC4/ADR (△) cells. [<sup>3</sup>H]DNR concentration was 0.5 μM. (B) The DNR efflux rate ( $v_{\text{pump}}$ ) was determined from the accumulation data in A (normalized to [DNR]<sub>i</sub> of 0.5 μM). Assuming a Hill kinetic fit of the data, the curves (five independent experiments) were determined by least-squares analysis. [ATP]<sub>i</sub> at half-maximal  $v_{\text{pump}}$  are shown for each experiment by dotted lines.

depletion on DNR transport were not dependent on [DNR]<sub>i</sub> (see Fig. 3). In Fig. 4B,  $v_{\text{pump}}$  is shown as a function of [ATP]<sub>i</sub>. ATP “stimulated” the DNR  $v_{\text{pump}}$  with no further increase above 3 mM. As shown,  $v_{\text{pump}}$  varied somewhat in the different experiments, but  $v_{\text{pump}}$  was half-maximal at the same [ATP]<sub>i</sub> ( $1.5 \pm 0.2$  mM, five separate experiments). The S-shape of the curve may suggest that regulation by ATP is a cooperative process (Hill coefficient

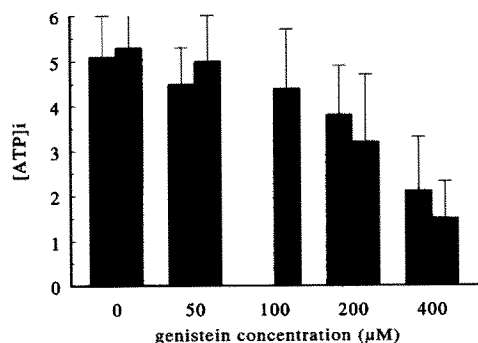


Fig. 5. Effect of genistein on cellular ATP content. GLC4 (closed bars) and GLC4/ADR cells (hatched bars) were incubated for 60 min at varying concentrations of genistein. Data are the means of at least three experiments.

$1.9 \pm 0.4$ ). However, [ATP] could not be measured at the site of the transporter molecules but instead [ATP]<sub>i</sub> was calculated, assuming a homogeneous concentration over the whole cell volume (see Discussion). Therefore, we feel that a detailed interpretation of the shape of the curve cannot be given without undue speculation.

#### Effects of genistein on [ATP]<sub>i</sub>

As shown in Fig. 5, genistein affected [ATP]<sub>i</sub> in GLC4 as well as GLC4/ADR cells. [ATP]<sub>i</sub> was decreased by genistein in a concentration-dependent manner: incubation with 50 μM had no effect on [ATP]<sub>i</sub> while 400 μM depleted [ATP]<sub>i</sub> to 1.5 mM in both sensitive and resistant GLC4 cells. Cellular ATP levels were decreased with more than 50% by 400 μM genistein in several (7 out of 10) other drug-sensitive and drug-resistant cell lines (data not shown).

#### DISCUSSION

Reduced cellular cytostatic drug accumulation due to overexpression of Pgp is often the underlying basis for MDR in tumor cells. In this study we report on the pharmacological characterization of a DNR transporter not encoded by *MDR1*. Characteristics for the existence of *protein* mediated, *energy-dependent* outward drug transport are: (1) transport of

drugs against a concentration gradient resulting in a decreased drug concentration at target; (2) inhibition of the enhanced drug efflux by inhibition of energy metabolism; (3) saturation of transport activity at high drug concentrations; and (4) competition of drug transport by structural analogs or other substrates for the transporter. We have shown previously that DNR accumulation in GLC4/ADR cells, which overexpress the *MRP* gene, was decreased against a concentration gradient due to an enhanced, energy-dependent extrusion of DNR [9]. By studying DNR accumulation in intact non-Pgp MDR GLC4/ADR cells we have been able to establish the following points: (1) the active transport of DNR appeared to be a saturable process with an apparent  $K_m$  value of DNR of 1.4  $\mu\text{M}$ ; (2) DNR transport was dependent on cellular ATP concentrations in the millimolar range; and (3) evidence for competitive inhibition of active DNR transport by genistein was provided. Thus our findings in the non-Pgp MDR GLC4/ADR cells are all consistent with the view that decreased DNR accumulation in GLC4/ADR cells is caused by a protein mediated, ATP-dependent DNR efflux. The transporter protein is most likely the 190 kDa, *MRP* encoded protein overexpressed in these cells [16, 22].

It has been demonstrated herein that genistein, which had previously been shown to reverse decreased DNR accumulation in non-Pgp (among them GLC4/ADR) but not in Pgp MDR cells [18], is a competitive inhibitor of DNR transport in GLC4/ADR cells (Fig 3B, Table 1). Verapamil and cyclosporin A, modulators of Pgp MDR, have been shown to interact competitively with Vinca alkaloids in binding to Pgp [24, 34]. However, inhibition by reversing agents has not been shown to have a competitive kinetic character in either case [25, 26]. Many but not all of these agents are transported by Pgp [25, 27, 28]. Further studies should establish whether genistein itself is transported and/or whether it binds at the DNR binding site to the drug transporter in GLC4/ADR cells.

Another significant aspect of the action of genistein is its effect on  $[\text{ATP}]_i$  (Fig. 5). In early studies on flavonoids, quercetin was shown to inhibit glycolysis and lactate transport in Ehrlich ascites tumor cells [29]. Recently genistein (400  $\mu\text{M}$ ) was shown to inhibit insulin-stimulated glucose transport by 50% and glucose oxidation completely in rat adipocytes [30, 31]. According to Fig. 4, the ATP depletion to  $\approx 70\%$  of normal ATP levels by 200  $\mu\text{M}$  genistein is too small to account for the increase in DNR accumulation in GLC4/ADR cells. Indeed, the competitive kinetics of genistein in Fig. 3 and Table 1 indicate that inhibition of DNR transport by genistein at concentrations  $\leq 200 \mu\text{M}$  is not caused by ATP depletion. This was also confirmed by performing a time-course experiment: genistein exhibited its effects on DNR accumulation within 5 min while energy depletion occurred after more than 15 min (data not shown). However, ATP depletion (to 1.5 mM) caused by a higher concentration of genistein (400  $\mu\text{M}$ ) is likely to affect DNR accumulation. Such an inhibition of ATP synthesis by high genistein concentrations implies that genistein in these concentrations should also affect decreased

DNR accumulation in Pgp MDR cells if DNR transport mediated by Pgp had dependence on ATP similar to that of DNR transport in GLC4/ADR cells. Indeed in Pgp MDR KB8-5 cells at 400  $\mu\text{M}$  genistein DNR accumulation was increased concomitantly with a decrease in ATP level to 14% of control. In another Pgp MDR cell line, SW-1573/2R160,  $[\text{ATP}]_i$  was not decreased by 400  $\mu\text{M}$  genistein nor was DNR accumulation affected (data not shown). Thus, the effects of genistein (at concentrations  $> 200 \mu\text{M}$ ) on DNR accumulation in Pgp MDR cells are associated with a decrease in ATP concentration. Therefore, for detection of non-Pgp mediated MDR by means of a DNR accumulation assay, genistein could only be used at concentrations  $\leq 200 \mu\text{M}$ .

In early studies on Pgp function, it was shown that Pgp mediated drug transport was ATP dependent [32, 33]. ATP, but not the nonhydrolysable analogs, stimulated the binding of drugs to isolated plasma membrane vesicles [34] and Pgp was shown to contain ATPase activity itself [35–38], suggesting that ATP provides the energy source for transport. Furthermore, it was shown by reconstitution of purified Pgp in artificial membranes that ATP hydrolysis was stimulated by VBL and other substrates for Pgp [36]. However, in intact Pgp MDR cells, only an enhanced ATP consumption and increase in cellular lactate production was observed by resistance modifiers such as verapamil, but not by DNR or vincristine [39, 40]. It was suggested that the rapid turnover time for verapamil compared to DNR or vincristine might be the reason that an increase in energy production and consumption could be measured in intact cells [39].

Here, we extend these data on Pgp MDR cells to *MRP* overexpressing GLC4/ADR cells. Similar to Pgp MDR cells, no effect of DNR on cellular ATP levels was measured in GLC4/ADR cells. This could be expected since, assuming one molecule ATP consumed per molecule DNR transported, the maximal consumption of ATP due to DNR transport would be 200 pmol/ $10^6$  cells/min (Table 1). This is only 1% of the estimated cellular ATP turnover in these cells [41].

The results presented here are the first to show how the DNR transport in a non-Pgp MDR cell line depends on changes in intracellular ATP levels. In GLC4/ADR cells the DNR transport was “stimulated” by increasing  $[\text{ATP}]_i$  with half-maximal stimulation at 1.5 mM ATP (Fig 4B). However, for Pgp and other members of the ABC-transporter family in isolated membrane preparations, an affinity for ATP in the sub-millimolar range ( $\approx 300 \mu\text{M}$ ) was found [2, 34, 36, 38, 42]. Such a high affinity makes it very unlikely that modest depletion of cellular energy will affect the drug transport function of Pgp. Indeed colchicine uptake was increased in resistant  $\text{CH}^{\text{R}}\text{C4}$  cells only when ATP levels were below 0.5 mM ATP [32]. In contrast, earlier studies demonstrated that Pgp activity was already slightly impaired by omission of glucose from the medium [33] or by reduction of ATP levels to  $\approx 40\%$  of control ( $\approx 1$ –1.5 mM [39, 43]). A similar phenomenon was observed for two other plasma membrane enzymes, Na,K-ATPase and ATP-sensitive K-channels [44, 45]. Whereas ATP reductions to low millimolar concentrations affected the two just-named enzyme

activities in intact cells, saturation of enzyme activity in isolated membrane preparations occurred in the micromolar range [42, 46].

A possible explanation for the discrepancy in ATP requirement in intact cells and membrane preparations may be a lower submembranal ATP concentration than the value derived from whole cell measurements [45, 47]. Cellular ATP depletion might already affect enzyme activity of plasma membrane enzymes in the millimolar range as shown in hepatocytes [45]. It would be unlikely for the GLC4/ADR cells that such a compartmentation would result from inhibition of different ATP synthesis routes since ATP depletion due to DG or  $\text{NaN}_3$  did not have differing effects on DNR transport. However, if such an ATP compartmentation is a common feature in cells, this would mean that the  $[\text{ATP}]_i$  of 1.5 mM found at half-maximal stimulation of drug transport in GLC4/ADR cells (Fig. 4B) actually represents a much lower  $K_m$  for ATP in drug transport.

Thus, we found that a modest depletion of cellular energy impaired DNR transport activity in intact cells. This might be of interest considering ATP levels in tumors which are in general low (mean  $\approx 1$  mM) and heterogeneous ( $< 0.5$  mM–3 mM [48, 49]). Therefore, it is worth investigating whether combining cytotoxic drugs with agents that interfere with energy supply might play a role in overcoming MDR. Interestingly, it was shown that GLC4/ADR cells were more sensitive to inhibitors of the oxidative phosphorylation than GLC4 cells [41], whereas DG was shown to be more toxic in resistant Pgp MDR cell lines compared to the parental cells [50]. Since glycolysis is the main energy producing pathway in many tumors, one might consider using agents that interfere with glycolysis. It was demonstrated by monitoring ATP levels with  $^{31}\text{P}$ -NMR *in vivo* that treatment with DG caused a decrease in tumor ATP levels to 50% without affecting ATP levels in the brain [51]. Although it may be clear from the above-described examples that little is currently known about the use of agents that limit the ATP supply as modulators of cytotoxic drug action in tumor cells, these data emphasize the possibility of using inhibitors of energy production to modulate MDR.

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