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Downregulation of taurine uptake in multidrug resistant Ehrlich ascites tumor cells

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Summary. In daunorubicin resistant Ehrlich ascites tumor cells (DNR), the initial taurine uptake was reduced by 56% as compared to the parental, drug sensitive Ehrlich cells. Kinetic experiments indicated that taurine uptake in Ehrlich cells occurs via both high- and low-affinity transporters. The maximal rate constant for the initial taurine uptake was reduced by 45% (high-affinity system) and 49% (low affinity system) in the resistant subline whereas the affinity of the transporters to taurine was unchanged. By immunoblotting we identified 3 TauT protein bands in the 50–70 kDa region. A visible reduction in the intensity of the band with the lowest molecular weight was observed in resistant cells. Quantitative RT-PCR indicated a significant reduction in the amount of taurine transporter mRNA in the resistant cells. Drug resistance in DNR Ehrlich cells is associated with overexpression of the *mdr1* gene product P-glycoprotein (P-gp). Using 5 progressively DNR resistant Ehrlich cell sublines with different P-gp expression pattern no correlation between taurine uptake and P-gp expression was found. Taurine uptake in *MDR1* transfected NIH/3T3 mouse fibroblasts was in contrast to the findings in Ehrlich cells increased compared to the parental fibroblasts. It is concluded that the reduced taurine uptake in resistant Ehrlich cells reflects a down regulation of the taurine transporter at the mRNA and protein level and it is most probably not related to P-gp overexpression.

Keywords: Amino acids – P-glycoprotein – TauT – NIH/3T3 – Daunorubicin

Introduction

Ehrlich ascites tumor cells use taurine as an organic osmolyte in order to restore cell volume after osmotic perturbation (Lambert, 1998). Taurine is accumulated in Ehrlich cells via a Na^+- and Cl^--dependent process and it appears that Na^+ binding to the taurine transporter is required for initiation of the transport cycle. Cloning of the taurine transporter from MDCK cells

(Uchida et al., 1992), rat brain (Smith et al., 1992), mouse brain (Liu et al., 1992) and human thyroid cells (Jhiang et al., 1993) revealed a transport protein with an tissue specific theoretical molecular weight of about 65–70 kDa and with putative sites for protein kinase C (PKC) and cAMP mediated phosphorylation (Liu et al., 1992; Jhiang et al., 1993). Modulation of the phosphorylation of the taurine transporter affects the transport capacity as well as the affinity of the transporter to taurine. In Ehrlich cells it has been demonstrated that active taurine uptake is reduced following activation of PKC and inhibition of calyculin A sensitive phosphatases, whereas uptake is stimulated by an increase in the cellular cAMP concentration (Mollerup and Lambert, 1996). Taurine is released from Ehrlich cells following osmotic cell swelling via a taurine channel that requires synthesis of leukotrienes (LTD_4) via the phospholipase A_2 and 5-lipoxygenase pathway for activation (Lambert, 1998).

Multidrug resistance (MDR), i.e., the acquired resistance of tumor cells to a number of structurally and functionally unrelated chemotherapeutic agents, is considered to be the major cause of treatment failure in cancer patients. Resistance is often associated with overexpression of P-glycoprotein (P-gp) (Litman et al., 2001), which is a 170 kDa, ATP-dependent drug efflux pump. P-gp has a broad substrate specificity and mediates cross-resistance to major classes of chemotherapeutic agents including anthracyclines, vinka alkaloids, podophyllotoxins and taxanes by preventing intracellular accumulation of drugs (Litman et al., 2001). The P-gp is in humans encoded by the *MDR1* and *MDR2* genes and in rodents by *mdr1a*, *mdr1b* and *mdr2* (Gottesman and Pastan, 1993). Only the human *MDR1* and the corresponding rodent *mdr1a* and *mdr1b* are able to confer MDR. P-gp belongs to the ABC superfamily of proteins that have diverse functions (Higgins and Linton, 2001), e.g., CFTR is a Cl^--channel and a regulator of a number of other plasma membrane associated ion-channels (Schwiebert et al., 1999), SUR-1 forms complexes with and regulates Kir 6.2 potassium channels (Mikhailov et al., 2000) and MRP5 transports cGMP (Higgins and Linton, 2001). P-gp has in addition to its drug efflux function been assigned a role as a regulator of endogenous swelling activated Cl^- channels in some tissues (Bond et al., 1998; Vanoye et al., 1999). These authors also proposed that PKC-mediated phosphorylation of P-gp modulates the activity of the swelling activated Cl^- channels.

This study was initiated to see whether (i) active taurine uptake, expression of the gene for the taurine transporter and the phosphorylation pattern of the taurine transporter were affected in a anthracycline resistant P-gp expressing Ehrlich ascites tumor cell line, and if (ii) there was a correlation between P-gp expression and taurine uptake.

Materials and methods

Chemicals

[^{14}C]-Taurine, [^{3}H]-Methyl amino isobutyric acid (meAIB), and [^{3}H]-Polyethylene glycol (PEG) were purchased from NEN Life Science products, Inc. Growth media, antibiotics

and sera were from Life Technologies (Denmark). Primary P-gp C-219 mouse antibody was from Centocor Diagnostics Malcolm (PA). Secondary anti mouse/rabbit horseradish peroxidase-linked antibodies were from Amersham Pharmacia Biotech (Denmark). All other compounds were from Sigma Chemical Co. PCR primers for Mus cooki taurine/beta-alanine transporters were from DNA technology, Århus, Denmark.

Cell lines

Ehrlich ascites tumor cells: Six different lines of Ehrlich cells (hyperdiploid strains) were used: The parental, drug-sensitive cell line (EHR2) and five daunorubicin-resistant cell lines. All cell lines were maintained in NMRI/DBA mice by weekly intraperitoneal inoculation. The resistant cell lines were developed by treatment of the host mouse with daunorubicin 0.8 mg/kg 4 times weekly (EHR2/0.8) from 6–72 passages (P6, P12, P36 and P72) or 1.3 mg/kg (EHR2/1.3) for more than 300 passages (Litman et al., 1998). One week after inoculation the cells were harvested and transferred to isotonic standard medium containing the anticoagulant heparin (2,5 IU/ml). Cell were washed twice and resuspended in isotonic standard medium at cytocrit 4–6% and incubated for 30 min (37°C). The standard medium contained the following solutes (mM): 143 NaCl, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 1 $CaCl_2$, 3,3 MOPS, 3,3 TES, 5 HEPES (pH 7,4).

NIH/3T3 fibroblasts: Parental (3T3) and *MDR1*-transfected (3T3/MDR1) mouse fibroblasts were grown in DMEM containing L-glutamine (2 mM), glucose (4.5 g/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% calf serum (v/v). Incubation temperature was 37°C and CO_2 5%. Cells were spilt (1:16) twice a week by trypsination (0.25%). The 3T3/MDR1 cells were permanent transfected with the pHaMDR1/A retroviral vector carrying human *MDR1* cDNA and selected in 60 ng/ml colchicine (Pastan et al., 1988). Expression of the *MDR1* gene product was verified by western blot analysis, using mouse, monoclonal C219 as primary antibody, and rabbit peroxidase conjugated anti-mouse, as secondary antibody.

Estimation of cellular taurine and cation concentrations

Taurine concentration: 1 ml of the cell suspension was transferred to preweighed Eppendorf vials and cells were separated from the media by centrifugation (14,000 g/45 sec). The supernatant was removed by suction, the vials reweighed and the cell pellet homogenized in 0.5 ml 4% sulfosalicylic acid. After 10 min the homogenate was centrifuged in order to precipitate proteins. The supernatant was filtered (Millex 0.22 μm) and the taurine content in the filtrate was estimated by ortho-phtalaldehyde (OPA) derivatisation followed by reverse phase HPLC (Nucleosil column, Macherey-Nagel, C18, 250/4, 100–5). Elution of OPA derivatives was performed with acetonitrile in a 12.5 mM phosphate buffer (pH 7.2) using a flowrate of 1 ml/min and by increasing the acetonitrile concentration from 0% to 25% within 25 min and from 25% to 50% within the following 2 min. Taurine was eluated after 16–17 min and the taurine concentration in the supernatant was estimated from the absorption at 330 nm, using taurine as standard (Kramhøft et al., 1997). Cell density in mg cell wet weight·ml^{-1} suspension and in mg cell dry weight·ml^{-1} was estimated from 1 ml cell suspension as previously described (Lambert et al., 1989). Taurine concentration is given as mmoles·l cell water^{-1}, where cell water was estimated as the difference between wet and dry weight of the precipitate of 1 ml cell suspension.

Ion concentrations: The cells were separated from the suspension medium by centrifugation as described above and deproteinized with perchloric acid (PCA) (final concentration 7%). The cellular concentrations of Na^+ and K^+, given as mmoles·l cell water^{-1}, were assessed by atomic absorption flame photometry (Perkin Elmer atomic absorption spectrophotometer) as previously described (Lambert et al., 1989).

Estimation of the rate constant for the initial taurine and meAIB uptake

The initial taurine and meAIB uptake in Ehrlich cells was determined as described by Lambert and Hoffmann (1993). In brief, ^{14}C-taurine (0.047 μCi·ml^{-1}), final extracellular taurine concentration 10 μM, or ^{14}C-meAIB (0.047 μCi·ml^{-1}), final extracellular meAIB concentration 0.1 mM, was added to 6 ml cell suspensions (cytocrit 4–6%) at time zero and five samples (1 ml) were taken within the first 5 min (taurine)/2 min (meAIB) for estimation of cellular and extracellular ^{14}C-activity. Cellular amino acid activity (cpm·g dry wt^{-1}) was corrected for trapped extracellular medium using ^{3}H-PEG as marker. The rate constant (min^{-1}) for the initial amino acid influx was calculated as the slope of the plot A_c^t/A_m^{to} vs. time, where A_c^t is the cellular activity at time t (cpm·g dry wt^{-1}) and A_m^{to} (cpm·g medium^{-1}) is the extracellular activity at time zero. The initial unidirectional amino acid influx (pmol·mg dry wt^{-1}·min^{-1}) was found by multiplying the rate constant by the extracellular amino acid concentration.

Taurine uptake in NIH/3T3 fibroblasts was estimated on cells plated in 6-well Nunclon dishes (9.6 cm^2 per well) and grown to ≈80% confluence (4.6×10^{-5} cells/well). Cells were washed three times in NaCl medium containing (mM): 150 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.1 $MgSO_4$, and 10 HEPES, and the influx initiated by removal of excess NaCl medium from the cells and addition of 2 ml NaCl medium containing ^{14}C-taurine (0.19 μCi/ml, final extracellular taurine concentration ≈1.7 μM). Cells were incubated for 5 to 30 min and the influx terminated by removal of excess extracellular ^{14}C-taurine followed by addition of 2 ml ice cold 100 mM $MgCl_2$. $MgCl_2$ was rapidly removed and the cells lyzed/fixed by addition of 200 μl ethanol (96%). The ethanol was aspirated and the water soluble ^{14}C-taurine extracted by addition of 500 μl water. Cell mass was estimated by protein determination (Lowry et al., 1951) on 100 μl of the water fraction using bovine serum albumine as standard. The ^{14}C-taurine accumulated by the cells was estimated from the activity in the remaining 400 μl of the water fraction. Rate constants were calculated as the slope of the of A_{cell}^t vs. time, where A_{cell}^t is the cellular ^{14}C-taurine activity (cpm·μg protein^{-1}) at time t.

Membrane potential

The membrane potential was measured according to the method described in Lambert, Hoffmann and Jørgensen (1989), using cell suspensions with a cytocrit of 0.25% and the fluorescent dye 1,1′-dipropyloxa dicarbocyanine ($DiOC_3$-(5), final concentration 1.8 μM) and calibrating the fluorescence signal with cells suspended in Na^+-free K^+/choline medium. The fluorescence (*excitation*: 577; *emission*: 605) was measured on a PTI RatioMaster spectrophotometer.

Human taurine transporter (hTauT) antibody

Polyclonal antibodies were raised in rabbits against a 32 kDa fusion protein consisting of the 49 N-terminal amino acids of the human taurine transporter (hTauT) and the 6xHis dehydrofolate reductase (DHFR) moiety of the Qiagen pQE40 plasmid. The fusion protein (6xHis-DHFR-hTauT1-49) was purified by FPLC on an immobilized Ni^+-matrix to which the histidine-tag binds. The purified protein was mixed with complete Freunds adjuvant for the initial immunization and with incomplete Freunds adjuvant for the following immunizations. The IgG fraction was purified on a G column, and antibodies towards the 6xHis-DHFR part of the fusion protein was absorbed on a column with immobilized 6xHis-DHFR protein.

Immunoblotting

Ehrlich cells, suspended at a cytocrit of 8%, were resuspended and lysed in Tris (50 mM) / sucrose (150 mM) / EGTA (0.2 mM) buffer containing 1 mM phenylmethanesulfonyl

fluoride, 1 μM pepstatin A, 10 μM E-64 and 1 mM leupeptin. The disrupted cells were sonicated (2 $\times$ 10 min) on ice and centrifuged at 600 g for 10 min. Supernatants were transferred to new Eppendorf tubes, centrifuged at 120,000 g for 45 min (4°C), and the pellet resuspended and sonicated in the Tris/sucrose/EGTA medium. This centrifugation and resuspension was repeated and the final membrane preparation was stored at −80°C. That membrane proteins are present in the final membrane preparation is verified by the presence of P-glycoprotein in the membrane preparation from Ehrlich cells using the P-gp C-219 antibody (data not shown).

Mouse fibroblasts were grown to 80% confluency in 83 cm^2 flasks, washed 3 times in isotonic NaCl medium and lysed in 3 ml of a buffer containing Tris (50 mM)/NP-40 (1%)/SDS (0.1%)/NaCl (150 mM)/NaAzide (0.02%)/NaDeoxycholate (0.5%). All cell preparations were stored at −80°C.

Before SDS-PAGE the membrane fractions were diluted 1:1 with solubilisation buffer containing 62.5 mM Tris, 4% SDS, 18% Glycerol, 200 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 10 μM E-64 and 1 mM leupeptin. Equal amounts of membrane preparations (25 μg protein) were separated on an 11% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane using a semidry blotter. Membranes were blocked with dry milk and incubated in primary TauT rabbit antibody (1:1500) or primary P-gp C-219 mouse antibody (1:3000) overnight. The blot was then incubated with a secondary horseradish peroxidase-conjugated antibody for 2 h. The label was detected using enhanced chemiluminescence kit from Amersham-Pharmacia. Specificy of the TauT antibody was evaluated by blockade of the western signal by addition of the 6xHis-DHFR-1-49-hTau fusionprotein to the primary antibody before addition of the antibody to the membrane.

RNA purification, cDNA synthesis, and RT-PCR

Total RNA was purified by the method of Chomczynski and Sacchi (1987). RNA concentrations and purity were determined by spectrophotometer readings at 260 and 280 nm. cDNA synthesis and RT-PCR were performed as described in (Nielsen et al., 2000). In brief: RNA aliquots of 10 μg were DNAse treated in the presence of RNAguard RNase inhibitor for 20 min, and thereafter phenol-chloroform extracted and ethanol precipitated. Samples containing 5 μg DNAse treated total RNA, were reverse transcribed in reverse transcriptase buffer, with random hexanucleotides (10 μM), deoxynucleoties, RNAguard Rnase inhibitor (20 U) and reverse transcriptase (10 U AMV) for 60 min at 42°C in a total volume of 20 μl. These cDNA preparations were diluted 10 fold, and five 5 μl aliquots used for RT-PCR amplification. Amplification of mRNA for the household gene β-actin was used as control. Two sets of PCR primers for Mus cookii taurine/beta-alanine transporter mRNA were used: Primer set 1, mTauTsense (583–605) 5′ CTT TGT TCT CTG GCA TTG CGT AC, mTauTantisense (784–808) 5′ GAG ACC CAG TGA CTC TCG TTC CTA, and Primer set 2, mTauTsense (1494–1516) 5′ CTA AGG AAG GGT TAT CGT CGG G, mTauT antisense (1721–1743) 5′ GCC AAT CAT GTC CTC AAT ACC G. PCR primers for Beta-Actin: mBetaActin sense (206–228) 5′ TGT GAT GGT GGG AAT GGG TCA G, mBetaActin antisense (719–741) 5′ TTT GAT GTC ACG CAC GAT TTC C. Twenty-five μl PCR reactions were run on a Perkin Elmer 9600 Thermo Cycler: 35 cycles; 94°C 30 sec, 55°C 30 sec, 72°C 30 sec. The PCR buffer contained Tris/HCl 10 mM, KCl 50 mM, $MgCl_2$ 1.5 mM, gelatin 0.01% (w/v), Triton X-100 0.01% (v/v), 200 μM of each deoxynucleotide, 250 nM of each PCR primer, and 1 U Taq DNA polymerase. Ten μl of the PCR-reactions were run in 2% agarose-gels and visualized by ethidium fluorescence.

Semi quantitative determination of mRNA

mRNA was amplified for five different numbers of cycles: 15, 20, 25, 30 and 35. 10 μl was run in agarose-gels and visualized as above. The amounts of PCR fragments were

quantified with a Pharmacia ImageMaster equipment. Both the genes with altered expression and the household gene beta-actin were in the linear part of the exponential amplification between 25 and 35 cycles. The beta-actin curves were overlapping for all the cell lines and therefore the gene specific differences can be used as semi quantitative estimates. The 25 or 30 cycle's points were used for comparative mRNA measurements as described in (Nielsen et al., 2000).

Statistical evaluation

Data are shown as means ± SEM with the number of experiments indicated. Significance was tested by paired Student's t-test. Differences were considered significant at $p < 0.05$.

Results

Taurine uptake is reduced in Ehrlich cells resistant to the anthracycline daunorubicin

In order to investigate whether the initial taurine uptake and the cellular taurine content were affected in multidrug resistant cells we measured the initial taurine uptake by tracer technique and the cellular amino acid concentration by HPLC technique in parental Ehrlich cells (EHR2) and in Ehrlich cells resistant to the anthracycline daunorubicin (EHR2/1.3). We find that in resistant cells the rate constant for the initial taurine uptake is significantly reduced from $6.3 \pm 0.4\,min^{-1}$ ($n = 13$) in parental cells to $2.8 \pm 0.3\,min^{-1}$ ($n = 13$) in resistant cells ($p < 0.001$; Fig. 1, upper left panel & lower panel). The cellular taurine concentration is concomitantly reduced from 19 mM to 15 mM ($p < 0.001$, Table 1). The water content (ml·g dry wt^{-1}) is identical in parental and resistant cells (Table 1), i.e., the reduction in the taurine concentration is not due to change in cell volume. Ehrlich cells accumulate taurine via a high-affinity transporter that exerts high specificity to β-amino acids (Lambert, 1998). Neutral amino acids, on the other hand are accumulated via system A and system ASC (Bussolati et al., 2001). The initial uptake of meAIB, which is considered as model substrate for system A (Bussolati et al., 2001), is similar in magnitude in both cell lines, i.e., the rate constant was estimated at $17.3 \pm 3.0\,min^{-1}$ ($n = 4$) in parental cells and 20.5 ± 4.2 ($n = 4$) in resistant cells ($p > 0.2$; Fig. 1 upper, right panel & lower panel). The alanine concentration in parental and resistant cells is also identical (Table 1). Thus, the reduced taurine concentration and the reduced taurine uptake seem to reflect a specific adaptation of the taurine uptake in resistant Ehrlich cells.

The taurine uptake in Ehrlich cells has previously been shown to be reduced following reduction in the extracellular Na^+ concentration and following depolarization of the plasma membrane (Lambert & Hoffmann, 1993). From Table 1 it is seen that the cellular concentrations of K^+ and Na^+ are almost identical in parental and resistant cells. However, the resting membrane potential is significantly depolarized from −50 mV to −44 mV (Table 1). The electrochemical gradient for Na^+, calculated from the cellular and extracellular Na^+ concentration and the membrane potentials, is reduced from $-8.3\,kJ{\cdot}mole^{-1}$ in parental cells to $-7.61\,kJ{\cdot}mole^{-1}$ in the resistant Ehrlich cells. Assuming that Na^+:taurine coupling for activation of the

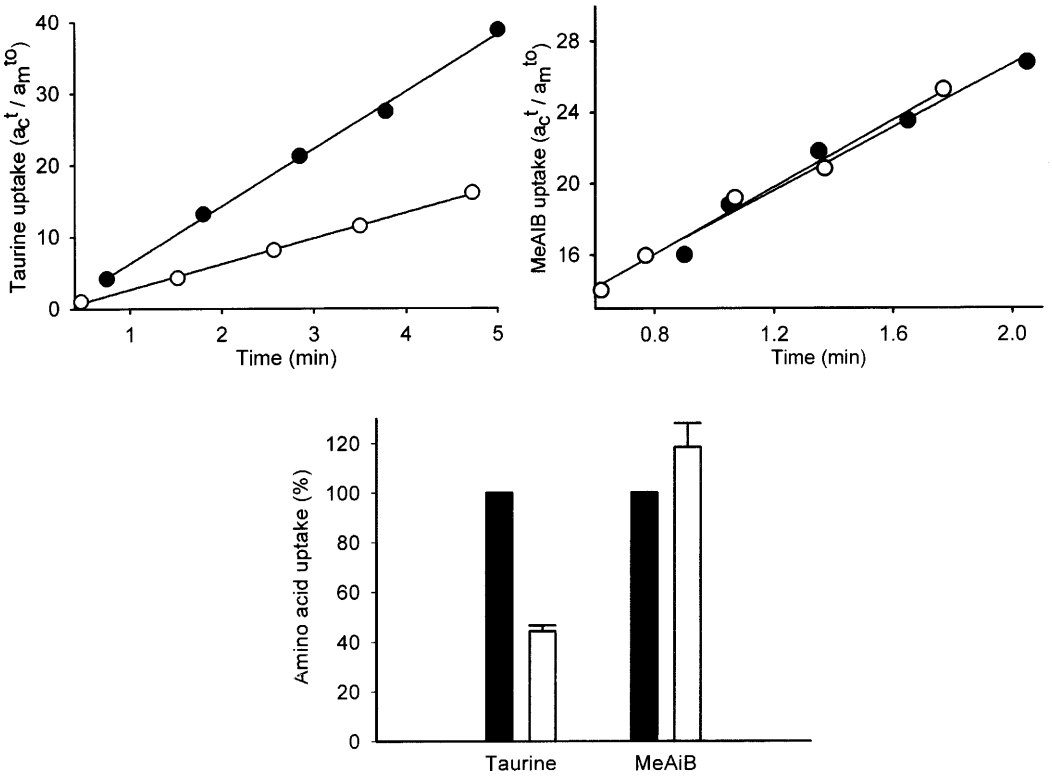

Fig. 1. Taurine uptake (upper, left panel) and meAIB uptake (upper, right panel) in parental Ehrlich cells (EHR2: closed symbol) and in multidrug resistant Ehrlich cells (EHR2/1.3: open symbol). ^{14}C-taurine (0.047 μCi/ml, final extracellular taurine concentration 10 μM, upper, left panel) or ^{14}C-meAIB (0.047 μCi/ml, final extracellular meAIB concentration 0.1 mM, upper, right panel) were added to cell suspensions (cytocrit 4–6%) at time zero and uptake was followed with time. Uptake is given as a_c^t / a_m^{to}, where a_c^t is the cellular activity at time t (cpm·g dry wt^{-1}) and a_m^{to} (cpm·g medium^{-1}) is the extracellular activity at time zero. Rate constants for the initial amino acid uptake were calculated as described in material and methods. Values for initial amino acid uptake (lower panel) in resistant cells (open bares) are shown relative to values in parental cells (closed bars). Values are given as mean ± SEM. The upper figures are representative experiments

taurine uptake is identical in parental and resistant Ehrlich cells, the reduction in the cellular taurine concentration seen in Table 1 could be secondary to the reduction in the electrochemical gradient for Na^+. On the other hand, the 6 mV depolarization of the plasma membrane would only be expected to reduce the initial taurine uptake maximally by 15% (Lambert and Hoffmann, 1993). Thus, the 56% reduction in the taurine uptake seen in resistant cells (Fig. 1) is not entirely related to the depolarization of the plasma membrane.

Expression of the taurine transporter is reduced in resistant Ehrlich cells

Taurine uptake in Ehrlich cells has previously been shown to take place via two saturable, Na^+ dependent systems (Lambert, 1984). Figure 2 (left panel)

Table 1. Cell volume, osmolyte concentration and membrane potential in parental and multidrug resistant Ehrlich cells. Cell volume is given in ml cellular water per g dry wt. Cation concentrations were determined by atomic absorption flame photometry. Amino acids were measured by OPA-derivatisation and reversed phase chromatography. Membrane potentials were estimated from the cellular/extracellular distribution of the fluorescent dye $DiOC_3$-(5). For details see Materials and methods. Values are ± SEM. The electrochemical gradient for Na^+ was estimated at $-8.3\,kJ{\cdot}mole^{-1}$ and $-7.61\,kJ{\cdot}mole^{-1}$ in parental and resistant Ehrlich cells, respectively

	Parental – EHR2	Resistant – EHR2/1.3	Reduction	p	n
Cell volume	3.02 ± 0.06 ml·g dry wt^{-1}	3.05 ± 0.07 ml·g dry wt^{-1}	–	0.35	18
Taurine	19.1 ± 0.8 mM	15.1 ± 0.8 mM	21%	<0.001	12
Alanine	9.9 ± 0.7 mM	10.4 ± 0.5 mM	–	0.25	12
Na^+	39 ± 5 mM	41 ± 6 mM	–	0.39	5
K^+	155 ± 5 mM	148 ± 4 mM	5%	0.029	4
E_m	−50.0 ± 3.8 mV	−44.2 ± 4.0 mV	12% depolarisation	<0.02	4

shows the saturation curves for the initial taurine influx in parental and resistant Ehrlich cells. The corresponding Eadie-Hoffstee plots reveal that the taurine uptake in parental as well as in resistant Ehrlich cells occurs via high- and low-affinity (H and L) transport systems (Fig. 2, right panel). Values for the maximal taurine uptake (J_{max}) and the Michaelis-Menten constant (K_{tau}) for the two transport systems were estimated from the Eadie-Hoffstee plots and included in Table 2. In accordance with previously published data (Lambert, 1984; Mollerup and Lambert, 1998) the high-affinity taurine transport system in parental Ehrlich cells has a low transport capacity (Table 2). The low-affinity taurine transport system in parental Ehrlich cells has a 2-fold larger transport capacity (Table 2). From Table 2 it is also seen that the J_{max} values for the low- and thigh-affinity systems are reduced by 49 and 45%, respectively, in resistant cells compared to parental cells. The K_{tau} values of the transporters, on the other hand, are similar, indicating that the affinity of the taurine transporters for taurine is identical in parental and resistant cells. In accordance with our findings the maximal velocity for taurine uptake in colon LoVo MDR cells, has recently been shown to be reduced compared the velocity in the non-MDR control cells (Wersinger et al., 2000). As the initial taurine influx experiments, presented in Fig. 1 are performed at 10μM extracellular taurine, i.e., at a concentration where the high-affinity transport system dominates, it is suggested that the reduced uptake in resistant Ehrlich cells reflects a reduced number of high-affinity transporters in the membrane.

In order to analyze the expression of the taurine transport protein we raised polyclonal antibodies against the 49 N-terminal amino acids of the human taurine transporter (hTauT). From Fig. 3 it is seen that the antibody recognizes three proteins with an estimated molecular masses of 50, 65 and 70 kDa in both parental and resistant Ehrlich cells. The molecular mass is consistent with the theoretical values (≈70 kDa) for the transporter (Jhiang et al., 1993). All bands disappeared when the primary hTauT antibody was blocked with the immunogene at a ratio 1:5 (data not shown), indicating that

Table 2. Rate constants for the initial taurine uptake and kinetic parameters for the high affinity / low affinity taurine transport in parental and multidrug resistant Ehrlich cells. The initial taurine influx was estimated as described in Fig. 1 (left panel). The maximal initial taurine influx (J_{max}) and the Michaelis-Menten constant (K_{tau}) were obtained from the saturation data in Fig. 2, which indicate that taurine uptake in Ehrlich cells is mediated by two saturable systems, i.e., a high affinity (H) and a low affinity (L) system. Values are given as means ± SEM

	Parental – EHR2	Resistant – EHR2/1.3	Reduction	n
Rate constant for the initial influx	6.3 ± 0.4 min^{-1}	2.8 ± 0.3 min^{-1}	56%	13
J_{max}^{L}	396 ± 26 pmol·mg dry wt^{-1}·min^{-1}	201 ± 23 pmol·mg dry wt^{-1}·min^{-1}	49%	4
K_{tau}^{L}	275 ± 37 μM	265 ± 20 μM	–	4
J_{max}^{H}	181 ± 4 pmol·mg dry wt^{-1}·min^{-1}	100 ± 16 pmol·mg dry wt^{-1}·min^{-1}	45%	4
K_{tau}^{H}	42 ± 13 μM	44 ± 10 μM	–	4

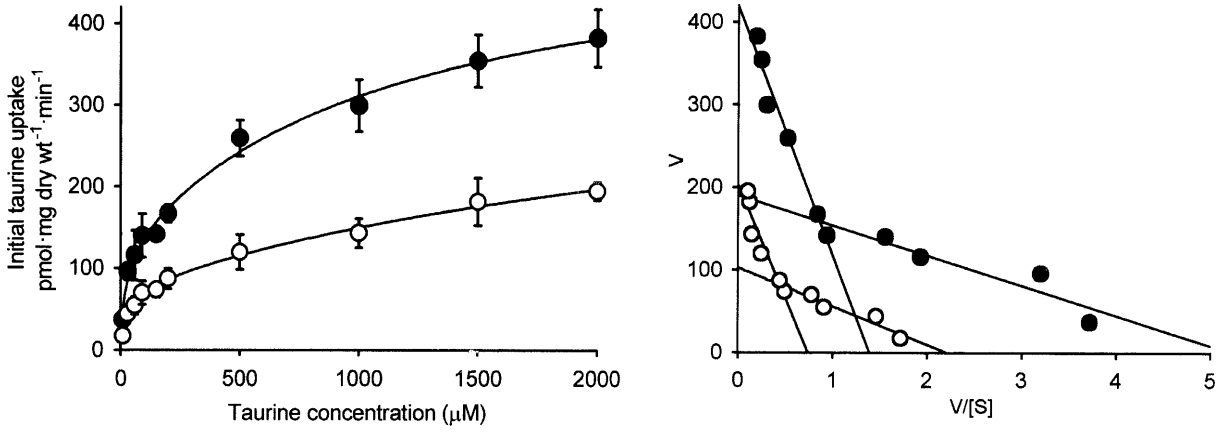

Fig. 2. Saturation curves for the initial taurine uptake in parental Ehrlich cells (EHR2: closed symbols) and in multidrug resistant Ehrlich cells (EHR2/1.3: open symbols). The taurine influx (V, pmol·mg dry wt·min^{-1}) was plotted versus the extracellular taurine concentration ([S], 10 μM–2 mM) (left panel). Eadie-Hoffstee plots (right panel), derived from the saturation data (left panel), indicate that taurine uptake consists of 2 saturable systems, one with high capacity and low affinity and one with low capacity and high-affinity. The curves in the saturation plot (left panel) are the best fit to the sum of 2 saturable systems ($y = ax/(b + x) + cx/(d + x)$) ($n = 4$). Maximal uptake velocity (J_{max}) and Michaelis Menten constants (K_{tau}) for the two saturable systems are calculated and included in Table 2. Values are given as mean ± SEM

all three proteins represent the taurine transporter. From Fig. 3 it is also seen that the intensity of the lower protein band is significantly reduced, whereas the intensity of the upper protein band is increased in resistant cells compared to parental cells. The observation that the intensity of the lower protein band (50 kDa) as well as the uptake capacity at low extracellular taurine concentration (J_{max}^{H}, Table 2) are concomitantly reduced could indicate that the 50 kDa protein band represents the high-affinity TauT, and that fewer copies are expressed in the resistant cells. Treating the Ehrlich cells with phorbol 12-

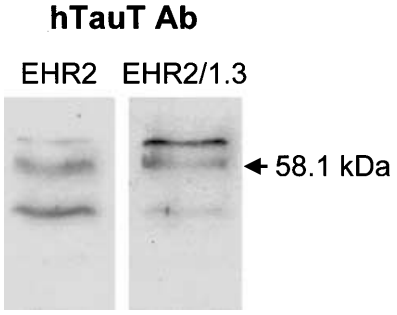

Fig. 3. Identification of the taurine transporter protein TauT in Ehrlich cells. Membrane preparation (20 μg protein) from both parental cells (*EHR2*, left lane) and resistant cells (*EHR2/1.3*, right lane) were subjected to SDS/PAGE (11% gel). The proteins were subsequently transferred to nitrocellulose membranes, incubated with rabbit anti-hTauT (primary antibody), blocked with dry milk and visualized with antirabbit horseradish peroxidase conjugated secondary antibody IgGs (chemiluminicence). Arrow indicates the 58,1 kDa molecular weight standard. The blots are representative of 3 sets of paired membrane preparations

myristate 13-acetate (PMA, 100 nM, 5 min), before extraction of the proteins, increased the intensity of the upper band (data not shown), indicating that expression of this protein is increased by activation of PKC by PMA. It is noted that our antibody also recognized two protein bands in the 25 to 30 kDa region which also disappeared when the primary TauT antibody was blocked with the immunogene. No difference in the protein band intensity between parental and resistant cells was observed. Han and coworkers (Han et al., 1996) also identified a 30 kDa protein using an antibody raised against the rat taurine transporter. Whether these low molecular proteins represent functional transporters, or spliced fractions, remains to be investigated. However, it should be noted that the 25 kDa band may represent recognition by the polyclonal antibody of the endogenous mouse DHFR.

As mentioned above PKC has been assigned a role in the regulation of taurine uptake in Ehrlich cells, i.e., stimulation of PKC by addition of PMA leads to reduction in the initial taurine uptake (Mollerup and Lambert, 1996). This is confirmed in Fig. 4, where it is shown that treating the parental Ehrlich cells with 40 nM PMA reduces the rate constant for the initial taurine uptake from 6.0 $\pm$ 1.0 to 5.3 $\pm$ 0.8 ($n = 3$). Whether the increased expression of the upper (70 kDa) protein seen in resistant cells (Fig. 3) and in PMA treated parental cells is involved in the concomitant reduction in taurine uptake remains to be established. From Fig. 4 it is seen that PMA addition reduces the rate constant from 3.1 $\pm$ 0.2 to 2.5 $\pm$ 0.1 in resistant cells ($n = 3$), i.e., the resistant cells are not less sensitive to PKC mediated reduction of taurine uptake. However, the rate constant for the initial taurine influx in PMA treated parental Ehrlich cells is significantly larger than the rate constant in resistant, control cells ($p < 0.03$). Thus, the reduced taurine influx seen in resistant cells seems mainly to be the result of a reduced number of high-affinity transporters in the plasma membrane although a reduction in transport activity due to PKC mediated phosphorylation cannot be excluded.

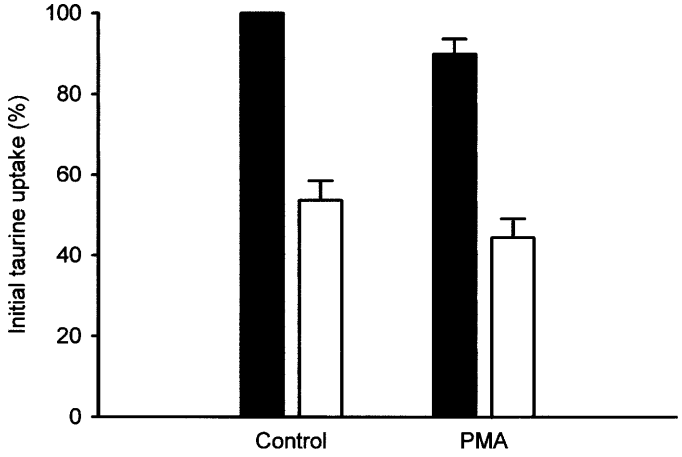

Fig. 4. Effect of phorbol 12-myristate 13-acetate (PMA) on the initial taurine uptake in parental Ehrlich cells (EHR2: closed bars) and resistant Ehrlich cells (EHR2/1.3: open bars). PMA (40 nM) was added 5 min. prior to the initiation of the influx experiment. Values in the figure are shown relative to values for parental cells. Taurine uptake in the resistant cells under control conditions was significant lower than taurine uptake in parental cells treated with PMA ($p < 0.03$). Values are mean ± SEM

Transcription of the TauT gene is reduced in resistant Ehrlich cells

In order to investigate whether the reduction in the expression of TauT protein in the resistant cells was due to regulation at the transcriptional level we measured the mRNA level of the parental and drug resistant EHR2/1.3 cells using RT-PCR and semiquantitative RT-PCR. The taurine transporter mRNA was detected with two sets of primers. By semiquantitative RT-PCR analysis a downregulation of the taurine transporter mRNA in the drug resistant cell line was seen (Fig. 5). The expression level in drug resistant Ehrlich cells is estimated at 40% of the level in parental Ehrlich cells. Thus, the reduction in the expression of the TauT transport protein seen in resistant Ehrlich cells (Fig. 3) seems to reflect a reduction in the transcription of the TauT gene.

The reduced taurine uptake in resistant Ehrlich cells is not correlated to p-gp expression

P-gp is a 170 kDa membrane protein that confers multidrug resistance to cells and tumors by active extrusion of chemotherapeutic drugs (Litman et al., 2001). P-gp has also been suggested to be a regulator of volume regulatory membrane transporters in various tissues (Bond et al., 1998). Parental Ehrlich cells as well as 5 progressively resistant Ehrlich cell lines including EHR2/1.3 have previosly been demonstrated by western blot analysis to express P-gp at different levels (Litman et al., 1995). We have used these cell lines to evaluate possible effects of P-gp expression on the initial taurine uptake. From Fig. 6 it is seen that parental cells express P-gp and that the amount of P-gp is increased

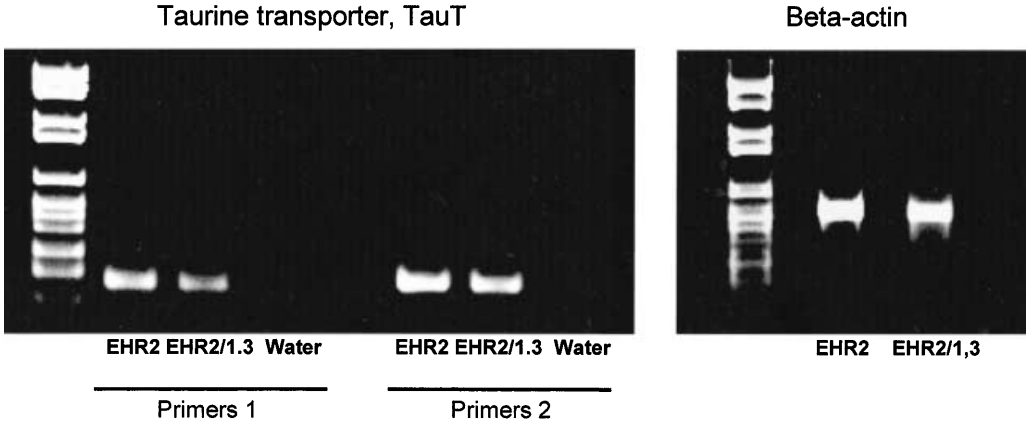

Fig. 5. Estimation of the expression of mRNA, that encodes for the taurin transporter TauT. The mRNA levels of the taurine transporter in parental EHR2 cells and multidrug resistant EHR2/1.3 cells were measured using RT-PCR and semiquantitative RT-PCR (left panel). The taurine transporter mRNA was detected with two sets of primers (see Materials and methods). The expression level in resistant cells is 40% of the level in parental cells. The mRNA level for the houshold gene for Beta-actin, measured by RT-PCR/semiquantitative RT-PCR (right panel), served as control

about 18 fold in the EHR2/1.3 cells. The taurine uptake in EHR2/1.3 is, on the other hand, reduced by 56% (Figs. 1 and 6, Table 2), indicating an inverse correlation between P-gp expression and taurine uptake. However, P-pg content as well as taurine uptake are initially increased in cells treated with daunorubicin for 6 to 12 passages (P6, P12) when compared to parental cells, whereas the initial taurine uptake in the resistant P36 cells is quantitatively similar to the uptake in parental cells ($P > 0.38$) even though the P-gp expression level is increased 24 fold (Fig. 6). Exposing the Ehrlich cells to daunorubicin for more than 12 passages (P12, P36, P72, EHR2/1.3) reduces the initial taurine uptake progressively ($P < 0.02$ for P72 and EHR2/1.3 compared to P6, see legend to Fig. 6), whereas the P-gp content seems to stabilize at a high level. The reduced taurine uptake in Ehrlich cells expressing equal but high levels of P-gp could be taken to indicate that downregulation of TauT is related to the MDR phenotype and independent of P-gp expression.

To further evaluate any effect of P-gp expression on taurine uptake we used mouse fibroblasts (NIH/3T3) overexpressing the MDR1 gene. From Fig. 7 it is seen that P-gp overexpression increases the taurine uptake significantly by $37 \pm 4\%$ ($P < 0.002$), i.e., from 37.3 ± 6.5 in parental fibroblasts to 58.3 ± 8.0 cpm·μg protein^{-1}·min^{-1} ($n = 4$) in MDR1-transfected fibroblasts. Thus, the initial uptake in MDR1 transfected fibroblasts are significantly higher than in the parental fibroblasts. The increase in uptake seen in MDR1 fibroblasts corresponds to the effect of moderate P-gp overexpression in Ehrlich cells (compare EHR2 to P6 and P12 in Fig. 6). The data in Figs 6 and 7 are taken to indicate that if there is an effect of P-gp on taurine uptake it is stimulatory. Thus, the reduced taurine uptake seen in EHR2/P72 and EHR2/1.3 is most probably not associated directly with the overexpression of P-gp.

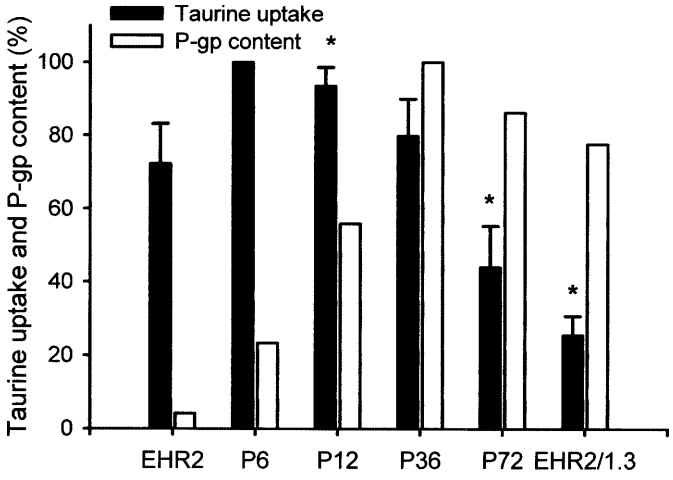

Fig. 6. P-glycoprotein (*P-gp*) expression and Taurine uptake. Five Ehrlich ascites cell sub lines from five progressive steps in the development of drug resistance (*P6, P12, P36, P72, EHR2/1.3*) were used together with the parental, drug-sensitive cell line (EHR2). All cell lines express different amounts of P-glycoprotein (open bars, data from Litman et al., 1995). Values for taurine uptake (closed bars) are given relative to values from P6. P-gp content is shown relative to P36. P6 and P36 were chosen because they had the highest taurine uptake and P-gp content, respectively. Asterisks (*) indicate that taurine uptake is significantly different from the uptake in parental cells ($p < 0.05$). The P-gp inhibitors / substrates verapamil (10μM), doxorubicin (10μM), vincristine (100 nM) and PSC-833 (100 nM) had no acute effect on taurine uptake when added 5 min. prior to the start of the experiments (data no shown). Values are given as mean ± SEM

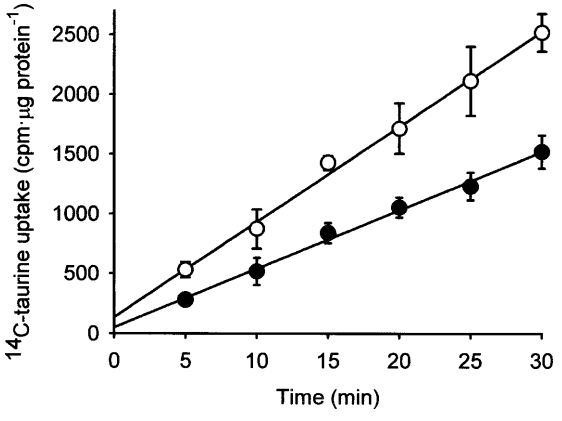

Fig. 7. Taurine uptake in parental and MDR1-transfected NIH 3T3 fibroblasts. Fibroblasts were plated in 6-well dishes and grown to ≈80% confluence. Cells were washed and the influx initiated by addition of 2 ml NaCl medium containing ^{14}C-taurine (0.19μCi·ml^{-1}), final taurine concentration was 1.7μM. The influx was terminated by removal of excess extracellular ^{14}C-taurine followed by addition of ice cold $MgCl_2$. The cellular ^{14}C-taurine activity was estimated on the water-soluble extract from the cells. Cell mass was estimated by protein determination and the ^{14}C-taurine accumulated by the cells (a_{cell}^t) given as cpm·μg protein^{-1}. Rate constants for taurine influx were calculated as the slope of the plot of a_{cell}^t vs. time. Values are given as mean ± SEM

Discussion

Reduction in taurine accumulation in resistant Ehrlich cells is secondary to the reduction in the transcription of the TauT gene

The data in Figs. 1 and 2 plus Table 2 clearly demonstrate that the taurine uptake in Ehrlich cells, resistant to the anthracycline daunorubicin (EHR2/1.3), is significantly reduced and that the reduction is a consequence of a reduction in the capacity (J_{max}) of the taurine transporting systems. The reduction in taurine uptake in EHR2/1.3 seems to reflect a specific adaptation of the taurine uptake in resistant cells as meAIB uptake via the ubiquitous A system for neutral amino acids is unaffected or even slightly stimulated (Fig. 1). Interestingly, the J_{max} for both the high-affinity as well as the low-affinity taurine uptake systems are reduced equally in resistant cells (≈50%, Table 2), which could indicate that the uptake systems either constitute the same transport protein in different conformations or that they are two separate proteins whose expression are regulated by the same cellular signals, e.g. transcription factors.

From the western blots (Fig. 3) it is seen that our antibody recognizes three bands in the 50–70 kDa range and that the intensity of the protein band with the lowest molecular mass (50 kDa) is reduced in resistant cells. As the level of mRNA encoding for TauT and J_{max} are concomitantly reduced in resistant cells (Fig. 5, Table 2) it is suggested that the 50 kDa protein represents the functional taurine transporter and that its expression is reduced in the resistant Ehrlich cells. On the other hand, the intensity of the 70 kDa protein is increased in resistant cells (Fig. 3). The intensity of the 70 kDa protein is also increased when PKC is activated in parental Ehrlich cells following treatment with PMA prior to the extraction of the proteins (data not shown). The activity of the taurine transporter in Ehrlich cells has previously been related to the phosphorylation state of the taurine transporter or a putative regulator (Mollerup and Lambert, 1996, 1998). However, it remains to be established whether the protein bands, recognized by our TauT antibody, actually represent different taurine transporters or one taurine transporter in different states of posttranslational modification.

The levels of Ca^{2+} activated PKC isoenzymes have been demonstrated to be elevated with the development of the MDR phenotype in, e.g., human MCF-7 breast cancer cells (Blobe et al., 1993; Ratnasinghe et al., 1998) and stimulation of PKC activity is known to reduce taurine uptake in human colon cells (Brandsch et al., 1993), Ehrlich cells (Mollerup and Lambert, 1996), rat astrocytes (Tchoumkeu-Nzouessa and Rebel, 1996) and MDCK cells (Han et al., 1999). Ser-322 is reported to be the critical site of PKC mediated phosphorylation in MDCK cells (Han et al., 1999). In the case of Ehrlich cells it has been shown that an increase in the phosphorylation of the taurine transporter or a putative regulator reduces (i) the maximal transport capacity of the high-affinity taurine transporter, (ii) its affinity to taurine as well as (iii) the Na^+:taurine coupling ratio for activation of the transport cycle (Mollerup and Lambert, 1998). As, the affinity of the taurine transporting systems is identical in parental and resistant Ehrlich cells (Table 2), it is suggested that no major

modification to the active transport protein in resistant cells has occurred. Furthermore, the sensitivity of resistant cells to PMA is similar to the sensitivity seen in parental cells (Fig. 4). Provided that the reduction in the initial taurine uptake seen after stimulation of parental cells with PMA (14%) represents the maximal reduction achievable by PKC-mediated phosphorylation, the reduction in taurine uptake seen in resistant cells (56%, Fig. 1) cannot be ascribed to a simple shift in phosphorylation of the taurine transporter or a putative regulator.

Wersinger and coworkers (2000) have demonstrated that the capacity (V_{max}) as well as the affinity of the taurine transporter to taurine were reduced in LoVo MDR cells and they suggested that the reduction could be due to either spatial hindrance of the incorporation of transporters in the plasma membrane or protein synthesis inhibition, caused by P-gp present in large numbers. In our model system such a conclusion cannot be supported as high initial taurine uptake coexists with high P-gp expression (see Fig. 6, P36). It is emphasized that treatment of the host mouse with daunorubicin for a relatively short period (EHR2/0.8: P6, P12) induces a massive increase in the expression of P-gp and a concomitant stimulation of the initial taurine uptake (Fig. 6). Similarly we find that NIH3T3 mouse fibroblasts, transfected with the human *MDR1* gene and selected for MDR with colchicine, also exhibit an increased taurine uptake (Fig. 7). On the other hand, treating the host mouse with daunorubicin for more than 36 passages (EHR2/0.8: P36, P72 and EHR/1.3) leads to a significant reduction in the initial taurine uptake, although the P-gp level remains high. Thus, the downregulation of the taurine uptake, seen after more than 36 passages with daunorubicin (P36, P72 and EHR2/1.3) could reflect the gradual development of further resistance mechanisms unrelated to P-gp expression. From Table 1 it is seen that the cellular taurine concentration is reduced by 20% in resistant cells compared to parental cells. Taurine has been assigned important cellular functions as an organic osmolyte, membrane stabilizer, antioxidant and regulator of the free cellular Ca^{2+} (Han et al., 2000a). The putative correlation between an increased drug resistance, reduced cellular taurine concentration and the cellular parameters affected by taurine is unknown.

Regulation of the TauT gene

Figures 3 and 5 indicate that transcription of the gene for the taurine transporter is reduced by about 60% in resistant Ehrlich cells and that the reduced TauT mRNA level is reflected in the amount of active transport proteins in the plasma membrane. Assuming that the fraction of mRNA translated into taurine transport proteins is similar in parental and resistant Ehrlich cells this reduction in transcription correlates with the reduction in the initial taurine uptake and the reduction in the transport capacities (J_{max}) of the taurine uptake systems. Han and coworkers (Han et al., 2000b) recently cloned and characterized the promoter region of the taurine transporter gene from rat kidney. They identified three consensus sites for the Sp1 transcription factor,

an overlapping site for WT-1/EGR-1/Sp1, two p53 half-sites, two estrogen receptor half-sites and a $TG_{22}/(A\text{-}C)_{22}$ repeat. SP1 is obligatory for basal activation of the TauT promoter, whereas TG repeat is critical for the full expression of the gene (Han et al., 2000b). The binding of both WT-1 and/or EGR-1 enhanced promotor activity (Han et al., 2000a). The renal taurine transporter promotor activity is, on the other hand, down-regulated by the tumor suppressor gene p53 (Han et al., 2000a). Daunorubicin induces diverse biochemical / biological effects, e.g. lipid peroxidation, topoisomerase II-dependent strand break, and intercalation into DNA double helix and a subsequent inhibition of DNA and RNA synthesis (Skovsgaard et al., 1994). The wild type p53 tumor suppressor is most probably inactive under normal cell conditions and not essential for normal performance of the cell (Oren, 1999). However, the sequence specific DNA binding activity of p53 is increased during genotoxic stress, e.g., treatment with anticancer drugs, which leads to modulation of the activity of several target genes (Muller et al., 1998) including activation of the human *MDR1* gene (Goldsmith et al., 1995) and mouse *mdr1* (Mathieu et al., 2001). The effect of p53 on the TauT gene is in contrast to its enhancing effect on the expression of P-gp. The observation that the expression of the mdr1 gene and the Taut gene are increased and decreased, respectively, in response to daunorubicin treatment of Ehrlich cells could suggest a role for p53 in the MDR phenotype in these cells and thereby explain the downregulation of the expression of the taurine transporter.

In conclusion, taurine uptake is reduced in daunorubicin resistant Ehrlich ascites tumor cells. The reduction reflects a reduced expression of the functional taurine transporter at the mRNA and the protein level. Neither phosphorylation of the taurine transporter or a putative regulator nor spatial hindrance of the incorporation of the transporter, caused by the presence of P-gp, seem to be involved in the reduction in taurine uptake.

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