BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport

Marc Heijn [a], Jan H. Hooijberg [a], George L. Scheffer [b], Gabór Szabó [c], Hans V. Westerhoff [d], Jan Lankelma [a,*]

[a] *Academic Hospital Vrije Universiteit, Department of Medical Oncology, Room BR232, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands*
[b] *Academic Hospital Vrije Universiteit, Department of Pathology, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands*
[c] *University Medical School of Debrecen, Department of Biophysics, P.O. Box 39, Debrecen, Hungary*
[d] *Vrije Universiteit, Department of Microphysiology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands*

Received 12 December 1996; accepted 9 January 1997

Abstract

We studied the ATP-dependent uptake of dinitrophenyl-glutathione (GS-DNP) into plasma membrane vesicles derived from parental GLC4 cells and from multidrug resistant GLC4/ADR cells. The latter have a high expression of the multidrug resistance protein (MRP). Uptake of GS-DNP into membrane vesicles from GLC4/ADR cells was highly stimulated by the addition of ATP, compared to the uptake into membrane vesicles from GLC4 cells. This ATP-dependent uptake into membrane vesicles from GLC4/ADR cells was saturable with a K_m of 1.2 ± 0.2 μM and a V_{max} of 560 ± 80 pmol/mg prot./min. ATP stimulated GS-DNP uptake with a K_m of 187 ± 4 μM. This uptake was specifically inhibited by a polyclonal serum raised against a fusion protein containing a segment of MRP. The ATP-dependent uptake of GS-DNP was not only inhibited by organic anions, such as oxidized glutathione (GSSG), methotrexate (MTX) and some bile acids, but also by non-anionic natural product drugs, such as anthracyclines, vinca alkaloids and etoposide (VP-16). Uptake of GSSG and MTX into membrane vesicles from GLC4/ADR cells could be stimulated by ATP. The ATP-dependent uptake of GSSG had a K_m of 43 ± 3 μM and a V_{max} of 900 ± 200 nmol/mg protein/min. The ATP-dependent uptake of GS-DNP seemed to be non-competitively inhibited by the anthracycline daunorubicin (DNR), whereas the ATP-dependent GSSG uptake seemed to be competitively inhibited by DNR. A substrate binding site on MRP is proposed that comprises a pocket in which both DNR and GS-DNP or GSSG bind in random order to different, only partly overlapping sites. In this pocket binding of a second compound is influenced by the compound which was bound first.

Keywords: Drug resistance, multiple; Biological transport; Adenosine triphosphate; Kinetics; Glutathione/aa; Daunorubicin

Abbreviations: DNR, daunorubicin; MRP, multidrug resistance protein; MDR, multidrug-resistance/resistant; MOAT, multispecific organic anion transporter; GS-DNP, 2,4-dinitrophenyl-S-glutathione; GSSG, oxidized glutathione; GSH, glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MTX, methotrexate; PMSF, phenylmethylsulfonyl fluoride

* Corresponding author. Fax: +31 20 4443844.

PII S0005-2736(97)00003-5

1. Introduction

Cancer cells selected for resistance against natural product drugs, such as doxorubicin, are often cross-resistant to a range of structurally and functionally unrelated drugs. This type of drug resistance is called multidrug resistance (MDR). The classical form of MDR is caused by the enhanced expression of the MDR1 gene, which encodes the P-glycoprotein (Pgp). Pgp is an ATP-dependent drug export pump located in the plasma membrane of various cell types [1]. Recently, a second ATP-dependent transporter of anti-tumor drugs has been identified in mammalian cells, the multidrug resistance protein (MRP) [2]. Transfection studies showed that MRP could confer resistance to a wide spectrum of natural product drugs [3,4]. MRP overexpressing cells are resistant to anthracyclines, to vinca alkaloids and especially to etoposide [4–8]. Expression of MRP did not confer resistance to cisplatin [5,6].

Isolated plasma membrane vesicles of MRP-transfected cells exhibited an ATP-dependent transport of glutathione(GS)-conjugates, such as leukotriene-C_4 and dinitrophenyl-glutathione (GS-DNP). Vesicles from untransfected cells exhibited only little such transport [9,10]. In addition, isolated plasma membrane vesicles from drug-resistant cell lines that overexpress MRP (GLC4/ADR and HL60/ADR) possessed an ATP-dependent transport system for GS-conjugates. This transport activity was almost absent in the parental human small-cell lung cancer cell line, GLC4, and the leukemic cell line HL60 [10,11].

The studies with isolated plasma membranes suggested that MRP functions as a transporter of negatively charged GS-conjugates. In experiments with intact cells, expression of MRP also conferred resistance to neutral and positively charged amphiphilic drugs. It has been suggested that these drugs are metabolized to anionic compounds that are transported by MRP. Reduction of the cellular glutathione (GSH) content by inhibiting its synthesis with buthionine sulfoxime, decreased drug resistance to daunorubicin (DNR) in MRP overexpressing cell lines [12–15]. This suggested that the conjugation with glutathione might be the relevant metabolism or that glutathione is involved in a (transient) complex formation. No such effect was seen in Pgp overexpressing cell lines [13–15]. Pgp transports unconjugated DNR [16]. However, such metabolites of, for instance, DNR have not been found in MRP drug-resistant cell lines and it has not yet been excluded that DNR itself is transported by MRP [17].

In order to gain more insight into the substrate specificity of MRP, we isolated plasma membrane vesicles of GLC4/ADR cells and studied the ATP-dependent uptake of GS-DNP into these vesicles. This transport could be inhibited by several anionic and cationic drugs.

2. Materials and methods

2.1. Chemicals

[^{3}H]GSH (1.6 TBq/mmol) was purchased from New England Nuclear (Dreieich, Germany). Dithiothreitol was removed from this solution of [^{3}H]GSH according to Butler et al. [18]. [^{3}H]GS-DNP was synthesized by conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to [^{3}H]GSH. To a solution of 20 μM [^{3}H]GSH and 1 mM CDNB, 1% NH_4OH was added. When the reaction was complete, acetic acid was added (5% (w/v) final concentration). [^{3}H]GS-DNP and [^{3}H]GSSG were separated on a reverse-phase C_{18} column (volume 1 ml, J.T. Baker, Phillipsburg, USA) equilibrated with 5% acetic acid. After addition of the reaction mixture (100 μl), the column was washed with 5% acetic acid. [^{3}H]GS-DNP was eluted with 30% (v/v) ethanol in 5% acetic acid. Unlabelled GS-DNP was purified by TLC [19]. [^{3}H]Methotrexate (866 GBq/mmol) was from Moravek Biochemicals (Brea, California, USA) and [^{3}H]DNR hydrochloride (163 GBq/mmol) from Dupont de Nemours ('s-Hertogenbosch, the Netherlands).

DNR hydrochloride was obtained from Specia (Paris, France), doxorubicin hydrochloride from Laboratoire Roger Bellon (France), and idarubicin (4′-demethoxy-daunorubicin) was kindly provided by Dr. J.W. Scheeren (Catholic University of Nijmegen, the Netherlands). Methotrexate was obtained from Pharmachemie BV (Haarlem, the Netherlands), taxol from Bristol-Myers Squibb BV (Woerden, the Netherlands), and VP-16 from TEVA Pharma (Mijdrecht, the Netherlands). ATP was obtained from Boehringer

Mannheim (Almere, the Netherlands). All other organic compounds were from Sigma (St. Louis, USA).

Cell culture media and supplements were obtained from Flow Laboratories (Irvine, UK). Fetal calf serum (FCS) was from Gibco (Paisley, UK).

2.2. Polyclonal antibodies

Polyclonal antibodies were obtained by immunizing mice, a rat and a guinea pig against bacterial fusion proteins containing segments of MRP. The MRP segments used were amino acids 192-360 (FP-I), 986-1204 (FP-V) and 1294-1430/1497-1531 (FP-III). The construction of the fusion proteins, the immunization protocol and the isolation of rat and mice anti-MRP monoclonal antibodies from these animals has been described by Flens et al. [20,21].

2.3. Cells

The GLC4 and GLC4/ADR human small cell lung cancer cell lines [22] were grown in RPMI medium supplemented with 7% heat-inactivated FCS at 37°C in a humidified atmosphere of 5–6% CO_2. The GLC4/ADR cell line was cultured in the presence of 1 μM of DOX until one week before plasma membrane isolation.

2.4. Plasma membrane vesicles

Plasma membrane vesicles derived from GLC4 and GLC4/ADR cells were isolated following a method adapted from Garrigos et al. [23]. Cells were harvested by centrifugation ($275 \times g$, 5 min) and washed once in phosphate-buffered saline. After incubating the cells in 100 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF and 50 mM Hepes/KOH (pH 7.4) for 1 h on ice, the cells were ultrasonicated at 20% of the maximum power of an M.S.E. Soniprep 150 sonicator for 3 bursts of 15 s. The homogenate (2.5 mg/ml) was centrifuged ($1500 \times g$, 10 min). The post-nuclear supernatant was layered on top of a 46% sucrose cushion. After centrifugation ($100\,000 \times g$, 60 min) the interface was removed and washed in 100 mM KCl and 50 mM Hepes/KOH (pH 7.4) ($100\,000 \times g$, 20 min) The final pellet was resuspended in 100 mM KCl and 50 mM Hepes/KOH (pH 7.4) by drawing the suspension 5 times through a 27-G needle. The final membrane preparations were stored at -80°C at a protein concentration of ~ 4 mg/ml.

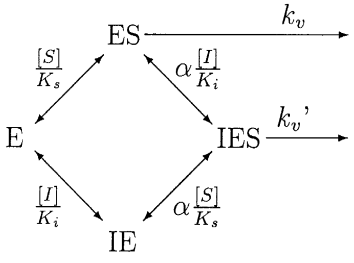
Marker enzyme activities were measured to determine enrichment of Na^+-K^+-ATPase (plasma membrane) [24], of esterase (endoplasmic reticulum) [25], of acid phosphatase (lysosomes) [26] and of glutamate dehydrogenase (mitochondria) [27]. Protein was determined by the Bio-Rad (München, Germany) protein assay.

2.5. Uptake studies

Uptake was measured by rapid filtration as previously described [28]. Plasma membrane vesicles ($\sim$ 0.25 mg protein/ml) were incubated for the indicated time at 37°C in 100 mM KCl, 50 mM Hepes/KOH (pH 7.4), 10 mM $MgCl_2$ with or without 2 mM ATP and with either [^{3}H]GS-DNP (1.6 kBq/pmol, 32 Bq/μl) or [^{3}H]GSSG (3.2 kBq/pmol, 32 Bq/μl) or [^{3}H]methotrexate (636 Bq/pmol, 190 Bq/μl). Incubation was stopped by adding 2.5 ml of ice-cold 100 mM KCl, 50 mM Hepes/KOH (pH 7.4) to 50 μl of the uptake medium. When the incubation time exceeded 5 min 50 μl was removed from a larger volume, incubated in a closed vial to prevent evaporation. After rapid filtration, filters (OE67, Schleicher and Schuell, Dassel, Germany) were washed twice with the stop buffer (2.5 ml). Both substrate and inhibitors were mixed with the vesicles at the start of the incubation. Polyclonal antibodies were preincubated on ice with the plasma membrane vesicles for at least 1 h, giving a 25 times dilution of the antiserum in the final incubation medium. The ATP-dependent uptake was determined by the difference between the uptake in the presence and that in the absence of ATP.

2.6. Calculations

The rate equation for the pocket model was derived from the following scheme.



In this scheme the binding site is represented by E.

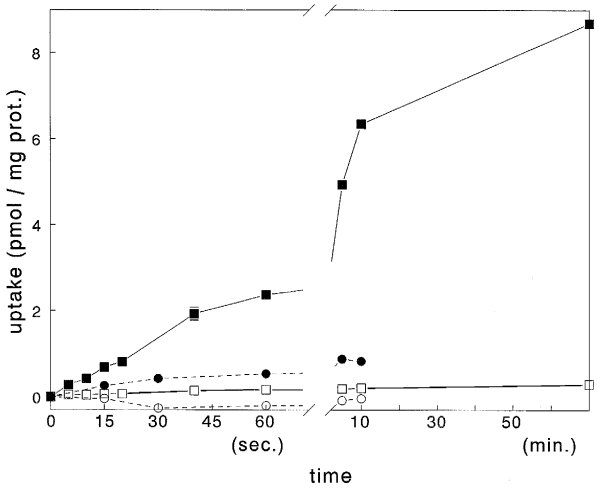


Fig. 1. ATP-dependent uptake of GS-DNP into membrane vesicles of parental GLC4 and multidrug resistant GLC4/ADR cells as function of time. Uptake of 10 nM [^{3}H]GS-DNP into membrane vesicles of GLC4 (dashed, ●, ○) and GLC4/ADR (□, ■) was measured in the presence (●, ■) or the absence (○, □) of ATP as described in Section 2. Data are from a representative experiment.

Binding of substrate S and inhibitor I to E occurs in random order. The relative amount of the different binding site forms (E, ES, IE and IES) depends on the concentrations of I and S and on the dissociation equilibrium constants of S (for ES and IES, K_s and K_s/α, respectively) and of I (for IE and IES, K_i and K_i/α, respectively). These constants certify microscopic reversibility. The binding co-operativity index α can be written as $e^{-w/kT}$ where w is the ''interactive free energy'' of S with I at the binding site (T is temperature in Kelvin and k the Boltzmann constant) [[29] (page 168)]. If $w \gg 0$ ($\alpha \to 0$) then binding is competitive, if $w > 0$ ($\alpha < 1$) then binding is negatively co-operative, if $w = 0$ ($\alpha = 1$) then binding is non-competitive and if $w < 0$ ($\alpha > 1$) then binding is positively co-operative. From ES and IES, S is transported at rates $[ES] \cdot k_v$ and $[IES] \cdot k'_v$, respectively. This transport rate was supposed to be much smaller than the dissociation rates of S or I from the binding site. This might be a valid assumption because for the related transporter P-glycoprotein it was estimated that the dissociation rate constant of daunorubicin was $1 \cdot 10^3$ s^{-1} [30] and that the catalytic rate constant (k_v) was 2–15 s^{-1} [31,32]. This catalytic rate constant is comparable with the estimated catalytic rate constant for MRP [33]. Calling $[E_{total}] \cdot k_v = V_{max}$ and $k'_v/k_v = f$ the rate equation of the transport of S becomes

$$v = \frac{V_{max}\left(\frac{[S]}{K_s} + f\alpha\frac{[S]}{K_s}\frac{[I]}{K_i}\right)}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \alpha\frac{[S]}{K_s}\frac{[I]}{K_i}}$$

In Section 3, data are presented as mean ± S.E.M. Signifance of differences ($P < 0.05$) between values were calculated with the Student t-test and between variations of the fits with an F-test.

3. Results

The final plasma membrane fraction had an enrichment of Na^+-K^+-ATPase by 5 times, of esterase by 1.4 times, of acid phosphatase by 2.5 times and of glutamate dehydrogenase by 0.3 times as compared to whole cells. In the assay, additional effective enrichment was obtained by only considering the ATP-dependent part of the uptake rate.

3.1. GS-DNP uptake

Uptake of GS-DNP into membrane vesicles of GLC4/ADR was stimulated by the addition of ATP

Table 1
Kinetic constants of the ATP-dependent GSDNP and GSSG uptake into GLC4/ADR membrane vesicles

	GS-DNP uptake		GSSG uptake		
K_m	1.2 ± 0.2	(n = 8)	43 ± 3	(n = 4)	μM
V_{max}	560 ± 80	(n = 8)	900 ± 200	(n = 4)	pmol/mg.protein/min
$K_{m,ATP}$	187 ± 4	(n = 4)			μM

The kinetic parameters were derived from n separate experiments as depicted in Fig. 2. The uptake of GS-DNP was measured in 15 s and of GSSG in 6 min. Data are presented as mean ± S.E.M.

(Fig. 1). ATP did not give such a stimulation of the GSDNP uptake into vesicles of the parental cell line, GLC4. The ATP-dependent uptake of GS-DNP into membrane vesicles of GLC4/ADR cells was linear with time for at least 15 s. This uptake was saturable for GS-DNP and ATP as shown in Fig. 2. The relation between the uptake rate of GS-DNP and the ATP or GS-DNP concentration fitted Michaelis-Menten kinetics. The kinetic constants which were obtained are summarized in Table 1.

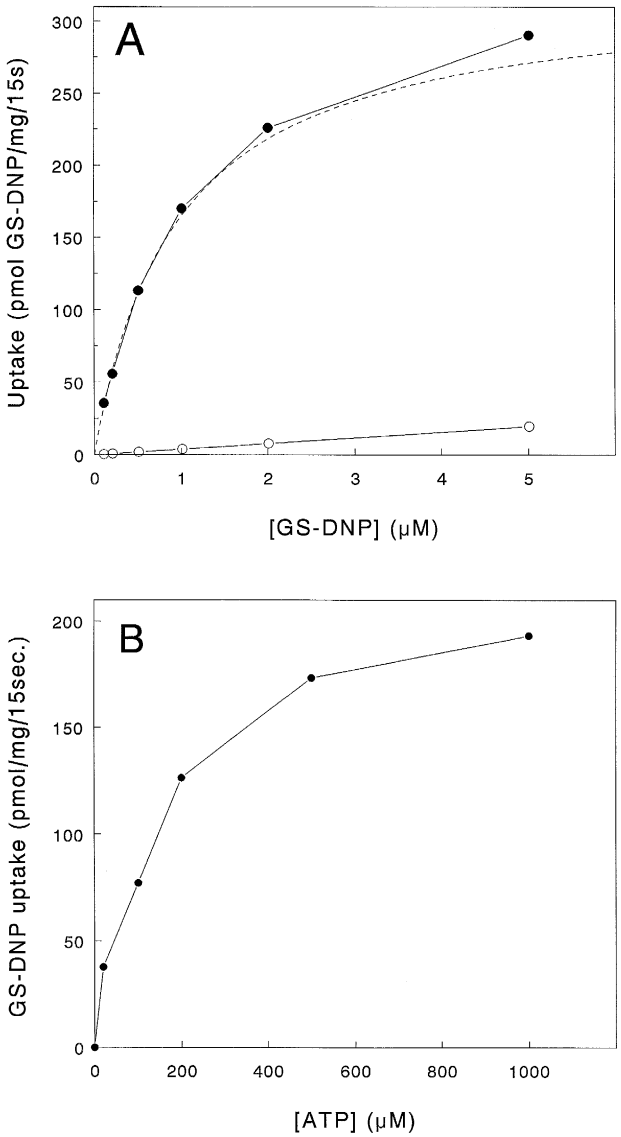


Fig. 2. ATP and GS-DNP concentration dependence of the ATP-dependent GS-DNP uptake into membrane vesicles of GLC4/ADR cells. Uptake of [^{3}H]GS-DNP into membrane vesicles of GLC4/ADR in 15 s was measured at different GS-DNP concentrations, ranging from 20 to 5000 nM GS-DNP, with (●) or without (○) 1 mM of ATP (A) and at different ATP concentrations, ranging from 20 to 1000 μM ATP, at 5 μM GS-DNP (B). The dashed line (upper panel) indicates the ATP-dependent uptake of GSDNP, obtained by subtracting the uptake of GS-DNP in the absence of ATP (lower curve) from the uptake in the presence of ATP (upper curve). Data are from a representative experiment.

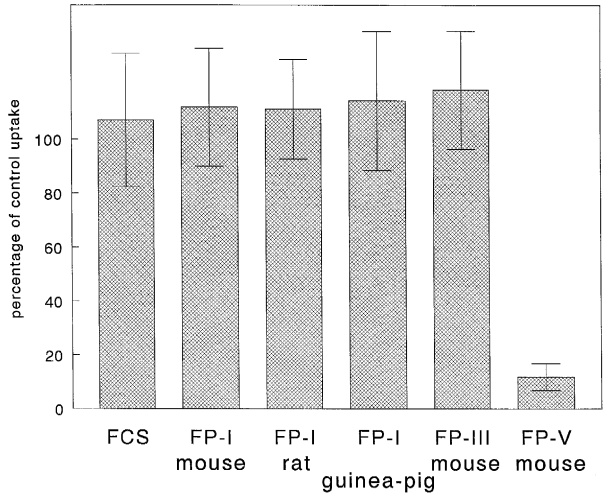


Fig. 3. Interaction of polyclonal antisera with the ATP-dependent GS-DNP uptake into membrane vesicles from GLC4/ADR cells. The ATP-dependent uptake in 15 s of 10 nM [^{3}H]GS-DNP into membrane vesicles from GLC4/ADR cells was measured with no additions (100%), fetal calf serum (FCS) and serum from mice, a rat and a guinea pig which had been immunized with the fusion proteins FP-I, FP-III and FP-V [20,21].

The ATP-dependent uptake of 10 nM of GS-DNP was studied in the presence and absence of several polyclonal antibodies raised against MRP as shown by Fig. 3. Only the polyclonal antiserum raised against FP-V inhibited the ATP-dependent GS-DNP uptake to $11 \pm 5\%$ compared to a control serum. This may very well indicate that this segment of MRP is important for MRP function.

The influence of several organic compounds on the ATP-dependent uptake of 10 nM GS-DNP is shown in Table 2. Organic anions, such as bile acids, probenecid, GSSG, MTX and calcein inhibited the GS-DNP transport. Taurine-conjugated bile acids were potent inhibitors. 3-Sulfated bile acids and more hydrophobic bile acids, such as (3-sulfated) taurolitocholic acid, were the most potent inhibitors. The cholephilic organic anion ICG also inhibited GS-DNP transport. In addition to these organic anions, anthracyclines, vinca alkaloids, VP16, taxol, as well as

Table 2
Inhibition of the ATP-dependent GS-DNP uptake into GLC4/ADR membrane vesicles by several organic compounds

	μM	percentage of control		μM	percentage of control
doxorubicin	10	32 ± 4	probenecid	500	55 ± 15
daunorubicin	10	31 ± 1	sulfinpyrazone	500	-1 ± 3
idarubicin	10	52 ± 2	calcein	500	40 ± 3
verapamil	50	97 ± 4	calcein-AM	10	44 ± 10
vincristine	50	46 ± 3	GSSG	100	36 ± 7
vinblastine	50	30 ± 6	ICG	10	34 ± 4
VP-16	10	50 ± 3	TC	10	72 ± 4
taxol	10	66 ± 8	STC	10	76 ± 4
methotrexate	10	82 ± 5	TLC	10	56 ± 5
rhodamine-123	10	104 ± 8	STLC	10	11 ± 2

Uptake in 15 s of 10 nM [^{3}H]GS-DNP into membrane vesicles of GLC4/ADR was measured in the presence and absence of 2 mM ATP. The ATP-dependent uptake of GS-DNP in the presence of the indicated organic compound was related to the ATP-dependent uptake of GS-DNP in its absence in three separate experiments. Control uptake was 0.76 ± 0.11 pmol GS-DNP/mg/protein/15 s. The abbreviations are: ICG, indocyanine green; TC, taurocholate; STC, 3-sulfated TC; TLC, taurolithocholate; STLC, 3-sulfated TLC. Data are presented as mean $\pm$ S.E.M. ($n \geq 3$).

calcein-AM inhibited the ATP-dependent transport of GS-DNP. At the concentrations in which they were used verapamil and rhodamine-123 did not inhibit GS-DNP uptake.

Uptake of the organic anions [^{3}H]MTX and [^{3}H]GSSG into membrane vesicles prepared from GLC4/ADR cells was also stimulated by ATP (Fig. 4). This stimulation was absent in membrane vesicles prepared from GLC4 cells. No significant stimulation of the uptake of the anthracycline [^{3}H]DNR by ATP was observed (data not shown). The kinetic constants of GSSG transport are shown in Table 1.

3.2. Inhibition mechanisms

We further studied the nature of the interference of DNR with GS-DNP uptake representative of the non-anionic cytostatic drugs [33]. Fig. 5A suggests that the inhibition by DNR is non-competitive (the slope reflecting the K_m for GS-DNP is not affected by DNR). DNR forms complexes with a variety of organic compounds [34]. The formation of a complex between GS-DNP and DNR which consequently decreases the free GS-DNP concentration could explain the lowering of GS-DNP transport activity by DNR. To exclude this possibility we tested whether GS-DNP could compete with DNA for the binding to DNR. If binding of DNR to GS-DNP is the cause of inhibition, the K_d of such a complex must be in the same range as the K_d of DNA (b.p.) and DNR (1–5 μM) [35]. GS-DNP alone does not quench the fluorescence of DNR. DNR is quenched upon binding to DNA but no decrease was seen in the fluorescence signal of DNR after the addition of an equal concentration of GS-DNP (1 μM). From this we conclude that sequestration of GS-DNP by DNR does not play a role. To further test this conclusion we studied the dose-dependent inhibition of GS-DNP transport by DNR in the presence of DNA (1 mg/ml). DNA buffers the free DNR concentration. The free DNR concentration in the presence of 1 mg/ml DNA at the same conditions of the uptake studies was determined as 5% of the total DNR concentration. To obtain the same inhibitory effect of DNR in the presence of 1 mg/ml DNA, we had to apply a 20 times higher DNR concentration.

We also studied inhibition of GSSG transport by DNR as shown in Fig. 5B. This figure shows that the inhibition by DNR of the GSSG transport, in contrast to that of GS-DNP, was competitive.

The inhibition by DNR of the GS-DNP transport fitted significantly better to a non-competitive type of interaction, whereas that of GSSG to a competitive type of interaction. In order to explain these different effects we propose a binding pocket that binds in random order DNR and GS-DNP or GSSG. When this model was applied to the data of the studies with GS-DNP transport the best fit (with a weighting

factor of $1/Y^2$) was obtained with a K_i, f and α of 0.95 ± 0.11 μM, 0.32 ± 0.11 and 0.88 ± 0.25 ($n = 6$), respectively. With the data of the transport studies with GSSG we obtained a K_i, f and α of 0.95 ± 0.32 μM, 0.68 ± 0.72 and 0.09 ± 0.14 ($n = 4$), respectively. The α for GS-DNP and DNR is ~ 1 which means non-competitive inhibition. The low α for GSSG and DNR shows strongly negatively co-operative (competitive) binding. Because of this low α, f could not be predicted accurately. The K_i of DNR found for GS-DNP transport was not significantly different from that found for GSSG transport. The α and f for GS-DNP and DNR predict that high concentrations of DNR cannot inhibit GS-DNP com-

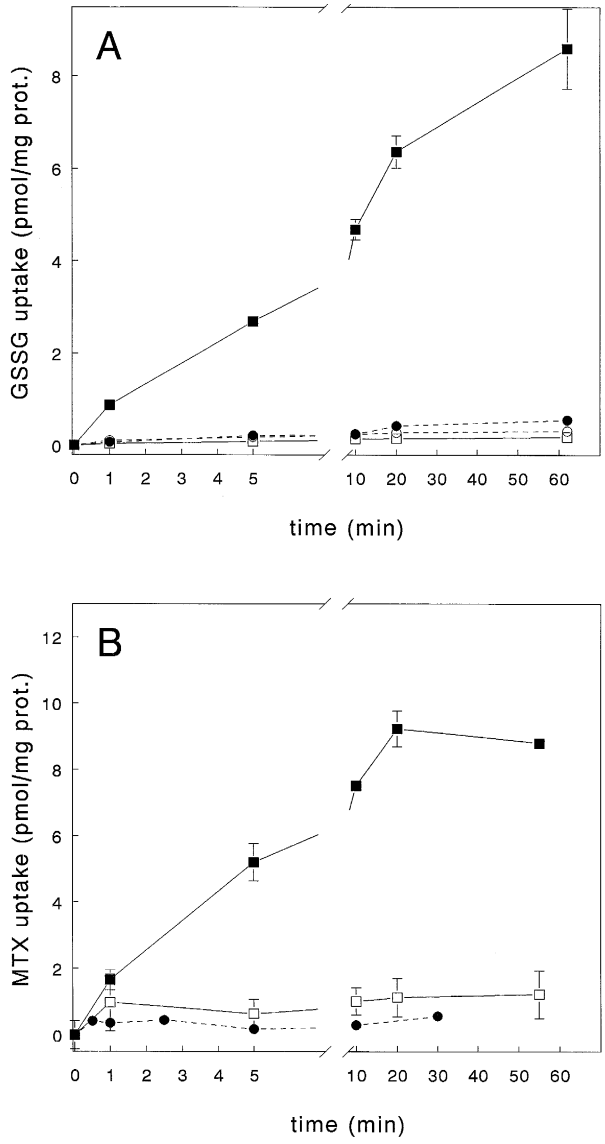


Fig. 4. Uptake of GSSG and MTX into membrane vesicles from GLC4/ADR cells. Uptake of 20 nM [^{3}H]GSSG (A) and 0.3 μM [^{3}H]MTX (B) into membrane vesicles GLC4 (dashed, ●, ○) and GLC4/ADR (□, ■) was measured in the presence (●, ■) or the absence (○, □) of 2 mM ATP as described in Section 2. Data are from a representative experiment.

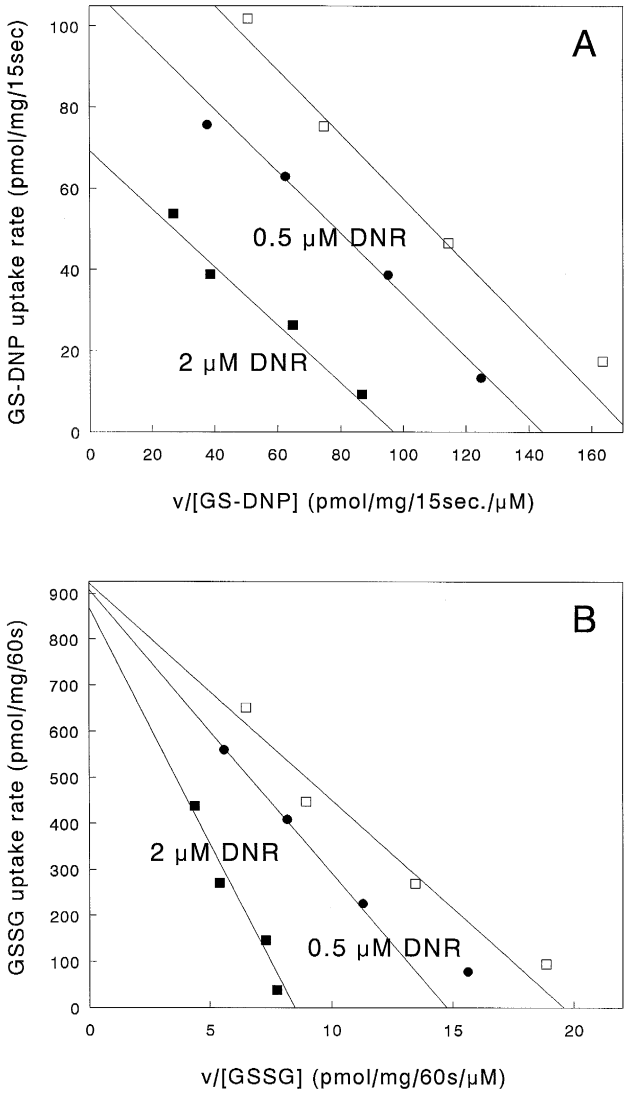


Fig. 5. Inhibition by daunorubicin of the ATP-dependent GS-DNP (A) and GSSG uptake (B) into membrane vesicles from GLC4/ADR cells. The uptake of [^{3}H]GS-DNP (A) and [^{3}H]GSSG (B) was measured at different substrate concentrations at different concentrations of daunorubicin (DNR), as indicated. The data of representative experiments are plotted in a Hofstee plot. The straight lines are the best fits by the pocket model.

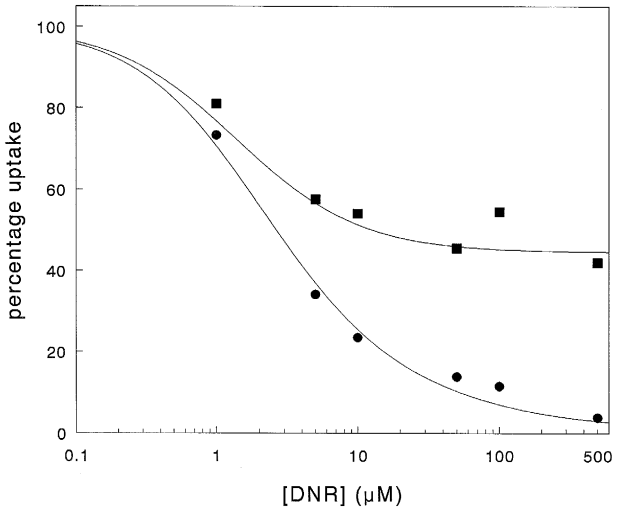


Fig. 6. Inhibition by high daunorubicin concentrations of the ATP-dependent GS-DNP and GSSG uptake into membrane vesicles from GLC4/ADR cells. The uptake of 10 nM [^{3}H]GS-DNP (■) and [^{3}H]GSSG (●) was measured at different concentrations of daunorubicin. Uptake without DNR was set at 100%.

pletely. To validate this model we tested this as shown in Fig. 6. Transport of GSSG was almost completely inhibited by 500 μM DNR, whereas GS-DNP transport could not be further inhibited till $\sim 40 \pm 20\%$ of the control uptake.

A different explanation may be the presence of two ATP-dependent transporters for GS-DNP, one sensitive to DNR and the other insensitive to DNR. The calculated K_i of DNR for this proposed DNR-sensitive GS-DNP transporter was 0.43 ± 0.13 μM, which is lower than for the GSSG-transporter.

4. Discussion

This study shows that the GS-conjugate GS-DNP is transported in an ATP-dependent manner into membrane vesicles of MRP overexpressing GLC4/ADR cells. This transport was almost absent from membrane vesicles derived from the drug-sensitive parental cell line, GLC4. Fig. 1 shows that at 10 nM GS-DNP more than 8 pmol of GS-DNP per mg protein could be taken up in the presence of ATP. Even if we overestimate the vesicular volume of the plasma membrane vesicles (10 μl/mg prot.) and assume that all these vesicles are inside-out plasma membranes, there would exist a concentration ratio for GS-DNP across the membrane which is more than 80. A significant part of this ratio may correspond to a ratio of free concentrations rather than due to intravesicular binding, as no such uptake was observed after long incubation in the absence of ATP.

These results confirm the findings of Müller et al. [10]. Here, the inhibition by the polyclonal antiserum raised against a fusion protein containing a segment of MRP provides additional evidence that the ATP-dependent transport of GS-DNP and other GS-conjugates is mediated by MRP in GLC4/ADR cells. Müller et al. found a higher K_m of 30 μM for GS-DNP transport into vesicles from GLC4/ADR cells [10]. It was shown that the ATP-dependent transport of GS-DNP across plasma membranes of other cell types had a K_m ranging from 2 μM to 300 μM (erythrocytes [36–38] and liver [39,40]). These differences were found when the same cell type was used, indicating that membrane preparation and experimental circumstances highly influence the measured apparent K_m. The presence of a competitive inhibitor could explain an increased apparent K_m.

Oxidized glutathione (GSSG) inhibited ATP-dependent GS-DNP transport. GSSG also inhibited leukotriene-C_4 transport, although to a lower extent; 0.5 mM of GSSG reduced the uptake to 48% [10]. We found that the uptake of GSSG could be stimulated by ATP in membrane vesicles from GLC4/ADR and not in those from the parent GLC4 cells. The calculated V_{max} of GSSG transport (0.9 nmol/mg/min) is in the same range as that of GS-DNP (0.56 nmol/mg/min). This suggests that GSSG, too, is transported by the system that transports GS-DNP, i.e., by MRP.

Other organic anions that are not GS-conjugates could also inhibit the GS-DNP uptake. The substrate specificity of MRP expressed in GLC4/ADR membrane vesicles resembles that of MOAT [28,41], with respect to organic anions. The uptake of the inhibitory organic anion and anticancer drug MTX, into GLC4/ADR membrane vesicles was stimulated by ATP. Such a stimulation was not observed for membrane vesicles of GLC4 cells; MTX also appears to be a substrate of MRP. The ATP-dependent transport system for MTX described in isolated inside-out plasma membrane vesicles of L1210 leukemia cells [42] may well be MRP proper.

The fluorescent organic anion calcein has been used as a probe of MRP activity in intact cells [15]. In the cellular studies the acetoxymethyl ester of calcein, calcein-AM, was given to cells. This lipophilic neutral compound diffuses into the cell where it is hydrolysed to fluorescent calcein. MRP expressing cells extrude the latter compound. The interpretation of the studies in intact cells was that mainly, but not exclusively, the free calcein is extruded by MRP [15]. In this study calcein-AM inhibited transport 30 times more strongly than calcein itself. As inhibition and transport may be only partly related, this does not exclude that the actual export rate of calcein-AM by MRP in intact cells is much slower than that of calcein.

In addition to calcein-AM many natural product drugs inhibited GS-DNP transport, although they were not anionic. The inhibition by vincristine and vinblastine confirms the results of Müller et al. [10]. In the quoted publication, these authors could not detect an inhibition by doxorubicin in their experimental conditions. However, when they applied our membrane preparation and experimental conditions, both DNR (10 μM) and doxorubicin (10 μM) reduced the uptake of leukotriene C_4 (2.3 nM) to 47% and 33%, respectively (personal communication by M. Müller). This is in line with the recent work of Loe et al. [43].

DNR appeared to compete with the binding of GSSG and inhibited GS-DNP transport in a non-competitive way. The different kinetic behavior of DNR towards GSSG and GS-DNP transport may suggest that GSSG and GS-DNP bind to different sites on MRP. However, it is more likely that both glutathione-conjugates bind to the same site. An alternative explanation is the presence of two ATP-dependent transporters for GS-DNP, one sensitive to DNR and the other insensitive to DNR. This is not very likely because (1) both transporters must have the same K_m for GS-DNP, (2) both transporters must be equally induced in GLC4/ADR cells and have a low expression in GLC4 cells. (3) The first transporter must transport GSSG with a K_m of 43 μM while the second must be a low affinity system for GSSG, whereas for GS-DNP it is a high affinity system. (4) Many compounds inhibit dose-dependently GS-DNP transport and can block transport completely (data not shown) and (5) the calculated K_i of DNR for the supposed DNR sensitive GS-DNP transporter is half the K_i for the GSSG-transporter. However, as explanation we propose a model including a binding pocket on MRP in which both DNR and the GS-conjugate bind (Fig. 7). In this model, the pocket is large enough to bind both GS-DNP and DNR. But if GSSG is present in the pocket, the binding of DNR is prevented, possibly by steric hindrance. Accordingly, when GS-conjugate and DNR are present in the pocket, GS-conjugate can still be transported. This may explain the contradictory finding that DNR not always gives an inhibition of GS-DNP transport. When the factor f and α equal one DNR will not give an inhibition of GS-DNP transport.

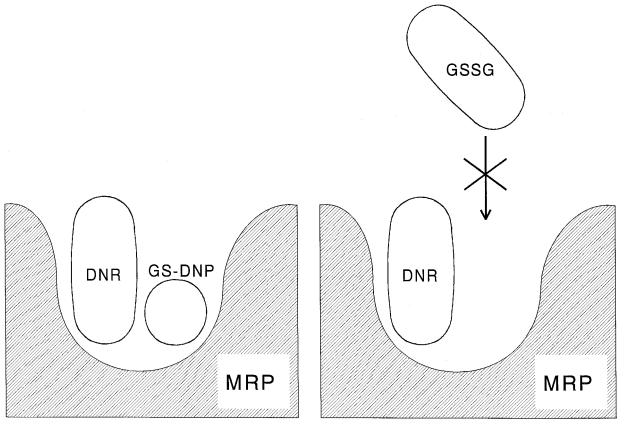


Fig. 7. Model of the binding pocket of MRP explaining non-competitive inhibition of GS-DNP transport and competitive inhibition of GSSG transport by DNR.

At the conditions described, uptake of DNR into membrane vesicles of GLC4/ADR cells could not be stimulated by ATP. This lack of ATP-stimulation could be caused by a high passive efflux rate of DNR compared with the active uptake rate, resulting in an insignificant accumulation. Recently, ATP-dependent transport of DNR in membrane vesicles from MRP overexpressing cells has been shown [44]. The analysis of the type of inhibition of GS-DNP and GSSG transport by DNR suggests that DNR binds to the same site as, or in the neighborhood of GSSG and GS-DNP. This makes it plausible that DNR is indeed (co-)transported by MRP. This model is in agreement with the results of Loe et al. [43]. They showed that ATP-dependent accumulation of vincristine in membrane vesicles of MRP expressing cells was stimulated by glutathione.

In this study we showed that MRP transports organic anions, such as GS-DNP, GSSG and MTX. This transport is specifically inhibited by a polyclonal antibody raised against a fusion protein containing a segment of MRP. At present we are characterizing a monoclonal antibody that also inhibits ATP-dependent transport of GS-DNP into membrane vesicles derived from GLC4/ADR cells. The kinetics of the inhibition of anthracyclines suggests the presence of a binding pocket in MRP. This model of a binding pocket may be of importance for understanding the broad substrate specificity of transporters such as MRP and P-glycoprotein. However, further detailed kinetic analysis of transport by these proteins is needed.

Acknowledgements

The authors thank Dr. Michael Müller (Department of Gastroenterology and Hepatology, University Hospital, Groningen, The Netherlands) for the uptake study with leukotriene C4, and Dr. Herbert M. Pinedo for financially supporting this work. This work was partly supported by the Dutch Cancer Society (Grant KWF-VU-95-933).

References

[1] Gottesman, M.M. and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427.
[2] Cole, S.P.C., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M.V. and Deeley, R.G. (1992) Science 258, 1650–1653.
[3] Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K.C., Cole, S.P.C. and Deeley, R.G. (1994) Cancer Res. 54, 357–361.
[4] Zaman, G.J.R., Flens, M.J., Van Leusden, M.R., De Haas, M., Mülder, H.S., Lankelma, J., Pinedo, H.M., Scheper, R.J., Baas, F., Broxterman, H.J. and Borst, P. (1994) Proc. Natl. Acad. Sci. USA 91, 8822–8826.
[5] Cole, S.P.C., Sparks, K.E., Fraser, K., Loe, D.W., Grant, C.E., Wilson, G.M. and Deeley, R.G. (1994) Cancer Res. 54, 5902–5910.
[6] Davey, R.A., Longhurst, T.J., Davey, M.W., Belov, L., Harvie, R.M., Hancox, D. and Wheeler, H. (1995) Leuk. Res. 19, 275–282.
[7] Coley, H.M., Workman, P. and Twentyman, P.R. (1991) Br. J. Cancer 63, 351–357.
[8] Baas, F., Jongsma, A.P.M., Broxterman, H.J., Arceci, R.J., Housman, D., Scheffer, G.L., Riethorst, A., Van Groenigen, M., Nieuwint, A.W.M. and Joenje, H. (1990) Cancer Res. 50, 5392–5398.
[9] Leier, I., Jedlitschky, G., Buchholz, U., Cole, S.P.C., Deeley, R.G. and Keppler, D. (1994) J. Biol. Chem. 269, 27807–27810.
[10] Müller, M., Meijer, C., Zaman, G.J.R., Borst, P., Scheper, R.J., Mulder, N.H., De Vries, E.G.E. and Jansen, P.L.M. (1994) Proc. Natl. Acad. Sci. USA 91, 13033–13037.
[11] Jedlitschky, G., Leier, I., Buchholz, U., Center, M. and Keppler, D. (1995) Cancer Res. 54, 4833–4836.
[12] Lutzky, J., Astor, M.B., Taub, R.N., Baker, M.A., Bhalla, K., Gervasoni, J.E., Rosado, M., Stewart, V., Krishna, S. and Hindenberg, A.A. (1989) Cancer Res. 49, 4120–4125.
[13] Zaman, G.J.R., Lankelma, J., Van Tellingen, O., Beijnen, J., Dekker, H., Paulusma, C., Oude Elferink, R.P.J., Baas, F. and Borst, P. (1995) Proc. Natl. Acad. Sci. USA 92, 7690–7694.
[14] Versantvoort, C.H.M., Broxterman, H.J., Bagrij, T., Scheper, R.J. and Twentyman, P.R. (1995) Br. J. Cancer 72, 82–89.
[15] Feller, N., Broxterman, H.J., Währer, D.C.R. and Pinedo, H.M. (1995) FEBS Lett. 368, 385–388.
[16] Guiral, M., Viratelle, O., Westerhoff, H.V. and Lankelma, J. (1994) FEBS Lett. 346, 141–145.
[17] Broxterman, H.J., Heijn, M. and Lankelma, J. (1996) J. Natl. Cancer Inst. 88, 466–467 (letter).
[18] Butler, J., Spielberg, S.P. and Schulman, J.D. (1976) Anal. Biochem. 75, 674–675.
[19] Awasthi, Y.C., Garg, H.S., Dao, D.D., Partridge, C.A. and Srivastava, S.K. (1981) Blood 58, 733–738.
[20] Flens, M.J., Izquierdo, M.A., Scheffer, G.L., Fritz, J.M., Meijer, C.J.L.M., Scheper, R.J. and Zaman, G.J.R. (1994) Cancer Res. 54, 4557–4563.
[21] Flens, M.J., Zaman, G.J.R., Van der Valk, P., Izquierdo, M.A., Schroeijers, A.B., Scheffer, G.L., Van der Groep, P., De Haas, M., Meijer, C.J.L.M. and Scheper, R.J. (1995) Am. J. Pathol. 148, 1237–1247.
[22] Meijer, C., Mulder, N.H., Timmer-Bosscha, H., Peters, W.H.M. and De Vries, E.G.E. (1991) Int. J. Cancer 49, 582–586.
[23] Garrigos, M., Belehradek, J., Lluis, M.M. and Orlowski, S. (1993) Biochem. Biophys. Res. Commun. 196, 1034–1041.
[24] Scharschmidt, B.F., Keeffe, E.B., Blankenship, N.M. and Ockner, R.K. (1979) J. Lab. Clin. Med. 93, 790–799.
[25] Wanders, R.J.A., Romeyn, G.J., Schutgens, R.B.H. and Tager, J.M. (1989) Biochem. Biophys. Res. Commun. 164, 550–555.
[26] Bergmeyer, H.U. (ed.) (1970) Methoden der enzymatischen Analyse Verlag Chemie Weinheim second edition, Vol. 1, pp. 457–458.
[27] Wanders, R.J.A., Van Roermund, C.W.T., De Vries, C.T., Van den Bosch, H., Schrakamp, G., Tager, J.M., Schram, A.W. and Schutgens, R.B.H. (1986) Clin. Chim. Acta 159, 1–10.
[28] Heijn, M., Oude Elferink, R.P.J. and Jansen, P.L.M. (1992) Am. J. Physiol. 262, C104–C110.

[29] Hill, T.L. (1985) Cooperativity Theory in Biochemistry, Steady-state and Equilibrium Systems. Springer, New York.
[30] Busche, R., Tümmler, B., Cano-Gauci, D.F. and Riordan, J.R. (1989) Eur. J. Biochem. 183, 189–197.
[31] Borgnia, M.J., Eytan, G.D. and Assaraf, Y.G. (1996) J. Biol. Chem. 271, 3163–3171.
[32] Broxterman, H.J. and Versantvoort, C.H.M. (1995) Pharmacology of drug transport in multidrug resistant tumor cells. In Alternative Mechanisms of Multidrug Resistance in Cancer (Kellen, J.A., ed.), Ch. 3, pp. 67–80, Birkhäuser, Boston.
[33] Broxterman, H.J., Giaccone, G. and Lankelma, J. (1995) Curr. Opin. Oncol. 7, 532–540.
[34] Dalmark, M. and Johansen, P. (1982) Mol. Pharmacol. 22, 158–165.
[35] Chaires, J.B., Dattagupta, N. and Crothers, D.M. (1983) Biochemistry 22, 284–294.
[36] Bartosz, G., Sies, H. and Akerboom, T.P.M. (1993) Biochem. J. 292, 171–174.
[37] Akerboom, T.P.M., Basas, R.D. and Sies, H. (1992) Biochim. Biophys. Acta 1103, 115–119.
[38] LaBelle, E.F., Singh, S.V., Srivastava, S.K. and Awasthi, Y.C. (1986) Biochem. J. 238, 443–449.
[39] Kobayashi, K., Sogame, Y., Hara, H. and Hayashi, K. (1990) J. Biol. Chem. 265, 7737–7741.
[40] Akerboom, T.P.M., Narayanaswami, V., Kunst, M. and Sies, H. (1991) J. Lipid Res. 266, 13147–13152.
[41] Oude Elferink, R.P.J., Ottenhoff, R., Radominska, A., Hofmann, A.F., Kuipers, F. and Jansen, P.L.M. (1991) Biochem. J. 274, 281–286.
[42] Schlemmer, S.R. and Sirotnak, F.M. (1992) J. Biol. Chem. 267, 14746–14752.
[43] Loe, D.W., Almquist, K.C., Deeley, R.G. and Cole, S.P.C. (1996) J. Biol. Chem. 271, 9675–9682.
[44] Paul, S., Breuninger, L.M., Tew, K.D., Shen, H.X. and Kruh, G.D. (1996) Proc. Natl. Acad. Sci. USA 93, 6929–6934.