ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 91–101

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane

Dietrun Kamp, Cees W.M. Haest *

Institut für Physiologie, Universitätsklinikum, Pauwelsstrasse 30, D-52057 Aachen, Germany

Received 7 January 1998; revised 5 March 1998; accepted 13 March 1998

Abstract

Phosphatidylserine (PS) containing a 7-nitrobenz-2-oxa-1,3-diazol-4-yl- (NBD-) hexanoyl residue, like native PS, preferentially distributes into the inner membrane leaflet of human erythrocytes. In the case of NBD-PS, this preference results from two opposite active processes, an inward translocation mediated by the aminophospholipid flippase and an outward translocation mediated by an ill-defined floppase. Selective inhibition of this floppase by alkylating reagents or cationic and anionic drugs increases the extent of accumulation of NBD-PS in the inner membrane leaflet from about 70% in control cells to about 90%. Different inhibitor sensitivities of the flippase and the floppase strongly suggest that both represent different entities. The floppase was characterized in further detail by comparing inhibitory effects of various compounds on this translocase with their effects on known primary active transport systems for amphiphilic compounds. The inhibitory effects of various drugs, glutathione conjugates and GSSG on the floppase activity closely correlate with those reported for the active transport by the multidrug resistance protein (MRP) while only poorly going parallel with those for the active transport by the low affinity pump for glutathione conjugates and the multidrug resistance MDR1 P-glycoprotein. The NBD-phospholipid floppase activity of the erythrocyte is thus probably a function of MRP.

Keywords: Erythrocyte membrane; NBD-phospholipid; Flippase; Floppase; Multidrug resistance (MDR); Multidrug resistance protein (MRP)

Abbreviations: ABC, ATP-binding cassette; CDNB, 1-chloro-2,4-dinitrobenzene; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione; EA-SG, glutathione conjugate of ethacrynic acid; GSH, glutathione; MDR, multidrug resistance; MOAT, multispecific organic anion transporter; MRP, multidrug resistance protein; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; 18:1,6-NBD-PC, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]*sn*-glycero-3-phosphocholine; 14:0,12-NBD-PC, 1-myristoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminolauroyl]-*sn*-glycero-3-phosphocholine; NBD-PS, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphoserine; PC, phosphatidylcholine; PS, phosphatidylserine; PCMBS, *p*-chloromercuriphenylsulfonate; TLCK, L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone; TPCK, L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone

* Corresponding author. Fax: +49-241-8888434; E-mail: cees@physiology.rwth-aachen.de

0005-2736/98/$19.00
PII S0005-2736(98)00049-2

1. Introduction

The inward translocation (flip) of phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer to the inner membrane leaflet of many plasma membranes is a fast, active process mediated by a flippase [1], meaning that the head group of the phospholipid approaches the binding site on the transporter from the lipid domain instead of the usual access of hydrophilic substrates from the aqueous medium [2]. This flippase has a high substrate specificity and only translocates the aminophospholipids, PE and PS [1]. It is a 115 kDa Mg^{2+}-dependent ATPase II that has been cloned recently [3]. In erythrocytes, protein mediation as well as Mg^{2+} and ATP dependence have also been reported for the outward translocation (flop) of newly synthesized PC [4], inner leaflet phospholipid species [5,6] and a variety of anionic phospholipid probes [7]. This floppase activity for phospholipid probes containing a 7-nitrobenz-2-oxa-1,3-diazol-4-yl- (NBD-) hexanoyl residue exhibits a low head group specificity [7] and has a considerably higher activation energy (104 kJ mol^{-1} [7]) than the flippase (31 kJ mol^{-1} [8]), which indicates the involvement of different mechanisms.

Further protein-mediated passive translocation pathways for phospholipid probes in erythrocytes concern the Ca^{2+}-dependent outward translocation of phospholipids [9] mediated by a 37 kDa protein that has been cloned recently [10] as well as the inward and outward translocation of mono- and di-anionic NBD-labeled phospholipids and long-chain amphiphilic anions, 5-(*N*-decyl)aminonaphtalene-2-sulfonate and 10-(α-naphtyl)-decyl-1-phosphate via the anion exchanger, band 3 protein [7,11–13]. In the latter case, a classical transporter that binds its substrate from the water phase also acts as a flippase that has to bind its substrate from the lipid phase because of very high membrane/water partition coefficients for these lipid probes [11–13].

In the present study, the floppase activity of the erythrocyte membrane was characterized functionally by comparing inhibitory effects of alkylating reagents and noncovalent inhibitors on this transporter with their effects on various other active transport systems for amphiphilic compounds, such as the aminophospholipid flippase, the glutathione conjugate transporters in the erythrocyte membrane [14–16] and the transporters responsible for drug resistance, i.e., the multidrug resistance protein (MRP [17,18]), which has recently identified in the erythrocyte membrane [17], and the MDR1 P-glycoprotein [19–22].

2. Materials and methods

2.1. Materials

Erythrocyte concentrates were obtained from the local blood bank and used within 10 days. Erythrocytes were isolated by centrifugation and washed three times with isotonic saline. The incubation medium (KNPS, pH 7.4) contained (mmol l^{-1}): KCl (90), NaCl (45), phosphate (12.5) and sucrose (44). 1-Oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine (18:1,6-NBD-PC) and 1-myristoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminolauroyl]-*sn*-glycero-3-phosphocholine (14:0,12-NBD-PC) were obtained from Avanti Polar Lipids (Alabaster, AL), dipyridamole, monensin, vinblastine, vincristine, oligomycin, daunomycin, staurosporine, indomethacin, quinidine, genistein, benzbromarone, prostaglandin A1, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Triton X-100, 1-chloro-2,4-dinitrobenzene (CDNB), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK), L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone (TLCK), *p*-chloromercuriphenylsulfonate (PCMBS) and ethacrynic acid were from Sigma (Deisenhofen), colchicine and phloretin were from Roth (Karlsruhe), salicylic acid was from Merck (Darmstadt), rose bengal was from Fluka (Neu-Ulm) and verapamil (Isoptin) was from Knoll (Ludwigshafen). 2,4-Dinitrophenyl-*S*-glutathione (DNP-SG) and the glutathione conjugate of ethacrynic acid (EA-SG) were prepared according to Saxena and Henderson [23]. NBD-labeled phosphatidylserine (PS) was prepared by transphosphatidylation of NBD-PC using phospholipase D as described before [7]. The leukotriene D_4 receptor antagonist MK 571 [24] was kindly provided by Dr. A.W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada).

2.2. Methods

2.2.1. Modification of erythrocytes

Erythrocytes were pretreated at 37°C with 0.03–1.0 mmol l^{-1} CDNB (30 min), 0.2 mmol l^{-1} TPCK, 0.5 mmol l^{-1} TLCK (15 min) or 0.1 mmol l^{-1} PCMBS (5 min) in KNPS followed by two washes with 0.2 g dl^{-1} albumin in KNPS and two washes with KNPS.

2.2.2. Measurement of the inward translocation (flip) of NBD-PS

After insertion of trace amounts of fluorescent NBD-phospholipid (8 nmol ml^{-1} packed cells) into the outer membrane leaflet of erythrocytes (7 min, 0°C), cells were washed once, resuspended in KNPS (hematocrit 10%) and incubated at 37°C. The time-dependent flip of the NBD-phospholipid to the inner membrane leaflet was measured by following the increase of fluorescence in the inner membrane leaflet, F_i, using the albumin extraction procedure [7]. Briefly, at various time periods 100 μl samples of the suspension were mixed with 550 μl medium containing 1.5 g dl^{-1} albumin and incubated (2 min, 21°C). After centrifugation, the albumin extraction was repeated once and cells washed with KNPS. Subsequently, cells were hemolyzed with 100 μl of water, lipids extracted by addition of 800 μl of isopropanol and the extract centrifuged. The fluorescence in the extracts was determined as described before [7]. The fluorescence in the inner leaflet was related to the total isopropanol-extractable fluorescence, F_{tot}, in the same amount of hemolyzed cells. An exponential function, $q[1-\exp(-k_1 \cdot t/q)]$, was fitted to the kinetic data of the non-extractable fractions (F_i/F_{tot}), where q represents the fraction of probe in the inner leaflet under stationary conditions and k_1 the rate constant for the unidirectional flip.

2.2.3. Measurement of the outward translocation (flop) of NBD-phospholipids

Following insertion of 15–20 nmol of NBD-phospholipid ml^{-1} packed cells and a flip period at 37°C of 45 min for NBD-PS, 90 min for 18:1,6-NBD-PC (in the presence of 1 mol l^{-1} ethanol to reversibly enhance its slow accumulation in the inner leaflet [7]) and 120 min for 14:0,12-NBD-PC, the probe was removed from the outer leaflet by two repetitive extractions (2 min, 21°C) with 20 volumes of KNPS containing albumin (1.5 g dl^{-1}). After washing of the cells with KNPS to remove albumin, the flop of the NBD-phospholipid at 37°C was followed by measuring the time-dependent decrease of fluorescence in the inner leaflet [7] using two methods. In the first method, the probe was allowed to equilibrate between the two membrane leaflets (37°C), i.e., the probe moves to the outer leaflet with a rate constant, k_{-1}, for the unidirectional flop and reaches a steady-state distribution between the two membrane leaflets with q the fraction of probe in the inner membrane leaflet under stationary conditions (equilibrative flop). To measure this flop, the time-dependent decrease of the probe in the inner leaflet was determined by two repetitive albumin extractions of 100-μl samples of the cell suspension as described above for the flip. An exponential curve, $(1-q)\cdot\exp[-k_{-1}\cdot t/(1-q)]+q$, was fitted to the kinetic data of the non-extractable fractions (F_i/F_{tot}).

In the second method, the flop was measured at 37°C in the presence of 0.75 g dl^{-1} albumin in the medium (1.5% hematocrit), which continuously removes the probe from the outer membrane leaflet (zero-trans flop). To this end, 750 μl samples of the cell suspension were centrifuged at various time periods of flop, cells were washed once with KNPS and lipids extracted to determine fluorescence. In this case q was set to zero.

2.2.4. Preparation of resealed ghosts and flop measurement

After insertion of the NBD-phospholipid probe into the membrane, 1 volume of erythrocytes was hemolyzed in 10 volumes of water (0°C) containing GSH (4–8 mmol l^{-1}) and ouabain (0.1 mmol l^{-1}). In certain preparations, DNP-SG, EA-SG or GSSG was present in the hemolyzing solution. Subsequently, ATP and $MgCl_2$ (3–5 mmol l^{-1}, final concentrations) were added and the hemolysate incubated for 10 min, followed by KCl (final concentration 150 mmol l^{-1}, 10 min 0°C). Ghosts were then resealed by incubation (15 min) at 37°C. Resealed ghosts were washed three times with KNPS. Following extraction of the phospholipid probe from the outer membrane leaflet by albumin, the flop was measured as described above for erythrocytes. The concentrations of DNP-SG and GSH within the resealed ghosts were determined after lysis of the ghosts, protein denatura-

tion and centrifugation by measuring the absorption of the supernatant at resp. 340 nm in absence and 413 nm in presence of 5,5-dithio-bis(2-nitrobenzoic acid).

2.2.5. Measurement of the outward transport of glutathione conjugates

Efflux of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) was measured according to Ref. [25]. Cells were pretreated (30 min, 37°C) with 1 mmol l^{-1} CDNB to convert GSH to DNP-SG, washed two times with KNPS containing 0.2 g dl^{-1} albumin and three times with KNPS. Subsequently, 1 volume of packed cells was resuspended in three volumes of KNPS and incubated at 37°C. At different time intervals, cell samples were sedimented by centrifugation and the supernatant was deproteinized by addition of $HClO_4$ followed by centrifugation. The amount of released 2,4-dinitrophenyl glutathione (DNP-SG) was then measured by the increase of the absorption of the supernatant at 340 nm.

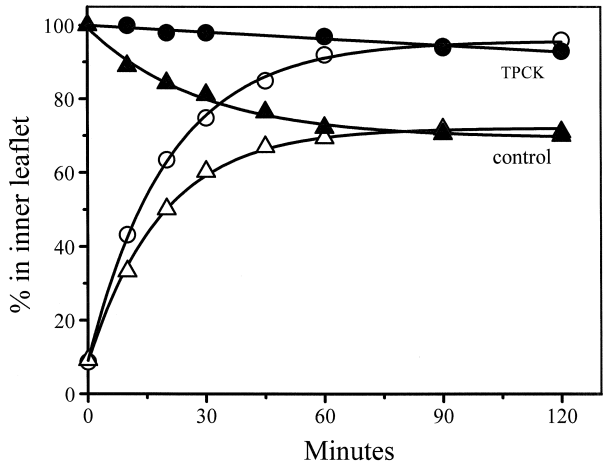

Fig. 1. Enhancement of translocation (37°C) of NBD-labeled PS from the outer to inner membrane leaflet (flip) as well as inhibition of its reverse translocation from the inner to the outer leaflet (flop) by a pretreatment (15 min, 37°C) of cells with 0.2 mmol l^{-1} TPCK. For details see Section 2. Open and closed triangles: flip and flop without pretreatment. Open and closed circles: flip and flop for TPCK-pretreated cells. Results from a characteristic flip and flop experiment out of a series of resp. 12 and 3.

3. Results and discussion

In previous studies on human erythrocytes [26], it has been shown that the rate of inward translocation (flip) of a fluorescent PS containing a 6-(NBD amino) hexanoyl residue at the 2-position of glycerol is slower than that of a spin-labeled PS probe with a 4-doxylpentanoyl residue [26]. Moreover, the stationary distribution of this NBD-PS proved to be significantly lower (somewhat below 80% inside [26]) than that of the spin-labeled PS (95% inside [26]) and endogenous PS (100% inside [27]). According to recent results from our laboratory [7,28], the stationary level of NBD-PS in the inner leaflet probably results from two opposite processes, an active inward translocation mediated by the aminophospholipid flippase and an outward translocation mediated by a (Mg^{2+}, ATP)-dependent floppase. In the present study, this concept was examined further and a functional characterization of the floppase was undertaken.

The time-dependent inward movement (flip) of NBD-PS to the inner membrane leaflet and its reverse movement to the outer leaflet (flop) at 37°C are shown in Fig. 1. By fitting exponential functions to the kinetic data, rate constants and fractions of the probe in the inner membrane leaflet under stationary conditions are obtained (see Section 2). The rate constants for the flip (k_1) and flop (k_{-1}) kinetics from a series of experiments are 0.028 ± 0.004 min^{-1} (half time = 17 min; $n = 17$) and 0.010 ± 0.002 min^{-1} (half time = 25 min; $n = 35$). The stationary levels of NBD-PS in the inner leaflet are $68.8 \pm 7.3\%$ ($n = 35$) following flip and $64.5 \pm 6.5\%$ ($n = 17$) following flop and thus comparable.

The above-mentioned higher stationary level of a spin-labeled PS probe is probably due to its 7-fold higher flip rate, $k_1 = 0.21$ min^{-1} [29] compared to 0.028 min^{-1} for NBD-PS, since the flop rates for the two probes are quite similar, $k_{-1} = 0.012$ [29] and 0.010 min^{-1}. In line with the idea that the levels of PS probes in the inner membrane leaflet result from the two opposite translocation processes via the flippase and the floppase, the ratios of the rate constants for the flip and flop of the spin-labeled PS and NBD-PS (17.5 and 2.8) correlate with the stationary distribution ratios of the probes between the inner and the outer membrane leaflet (19 and about 2.0).

The accumulation of NBD-PS in the inner leaflet increases upon a lowering of temperature. At 15°C,

the stationary level of NBD-PS in the inner membrane leaflet rises to 91% (data not shown). This can be explained by the lesser contribution of the floppase, at the lower temperature, to the stationary distribution of the probe. Floppase activity has a steeper temperature dependence ($E_a = 104$ kJ mol^{-1}; [7]) than the flippase ($E_a = 20$ kJ mol^{-1}, data not shown). Pursuing this argument, selective inhibition of either flippase or floppase activity should shift the stationary distribution of NBD-PS in favour of the outer or inner membrane leaflet.

In the course of our investigations this expectation turned out to be true. Several reagents were found to shift the stationary distribution of NBD-PS in favour of the inner membrane leaflet in parallel with an increase of the flip rate. A pretreatment of cells with the alkylating reagent [30] L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK; 0.2 mmol l^{-1}, 15 min, 37°C), known as a protease inhibitor, enhanced the flip rate by a factor 1.5 to $k_1 = 0.042 \pm 0.005$ min^{-1} ($n = 12$) and increased the accumulation of NBD-PS in the inner membrane leaflet (Fig. 1) from 69% to 94.1 ± 3.0% ($n = 12$). Similar results were obtained after a pretreatment of cells with 1-chloro-2,4-nitrobenzene (CDNB, 0.5–1.0 mmol l^{-1}). CDNB reacts with intracellular GSH catalyzed by glutathione-*S*-transferase and produces 2,4-dinitrophenyl-*S*-glutathione (DNP-SG [25]). The formation of this conjugate goes along with an increase of the fraction of NBD-PS in the inner membrane leaflet from 69 to 81.9 ± 4.7% ($n = 7$).

Besides these covalent modifications, treatment of erythrocytes with reversibly binding compounds such as verapamil (50 μmol l^{-1}; Fig. 2), dipyridamole, indomethacin or prostaglandin A1 (20 μmol l^{-1}) and daunomycin or oligomycin (30 μmol l^{-1}) increased the accumulation of NBD-PS in the inner membrane leaflet from 69 to 86–94%, ($n = 2$, data not shown).

These higher accumulations of NBD-PS in the inner leaflet by the various covalent and noncovalent reagents were paralleled by variable increases of the flip rate constant for NBD-PS (Table 1). Stimulation of the flippase by reversibly binding drugs is not responsible for the enhanced flip rate, since no significant flip enhancements by these compounds were observed in TPCK-pretreated cells (data not shown). Considering the composite origin of the stationary distribution of NBD-PS, the flip stimulations are

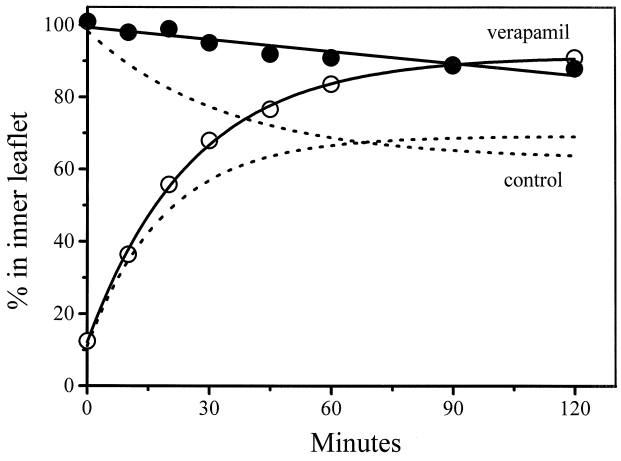

Fig. 2. Enhanced flip rate and accumulation in the inner membrane leaflet of NBD-PS (open circles) and simultaneous inhibition of its flop (solid circles) by addition of 50 μmol l^{-1} verapamil during the translocation measurement at 37°C. The dotted lines represent the flip and flop kinetics in the absence of verapamil (37°C). Results from a characteristic experiment out of a series of 3 at the same concentration.

likely to arise from an inhibitory effect on the flop process. This could indeed be demonstrated. The flop of NBD-PS proved to be strongly inhibited (95%, $n = 3$) by a pretreatment of cells with TPCK (Fig. 1), CDNB, and other alkylating reagents, L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone (TLCK) and ethacrynic acid (Table 1). Moreover, the flop was suppressed by the presence, during the measurement, of cationic drugs such as verapamil (Fig. 2), vinblastine, colchicine, vincristine, quinidine and staurosporine as well as of anionic drugs such as indomethacin, ethacrynic acid, monensin, benzbromarone, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and genistein (Table 1).

To substantiate that this inhibition of the flop process is really due to inhibition of the floppase rather than a stimulation of the flippase, the NBD-PS flop was measured in the presence of albumin in the medium (0.75 g dl^{-1}) at 1.5% hematocrit. This provides for a continuous extraction of the probe from the outer membrane leaflet, which keeps the concentration of the probe in this leaflet during the flop very low (zero-trans flop [28]) and thereby excludes contributions of a flippase-mediated inward movement of NBD-PS to the flop kinetics. As shown in Fig. 3, TPCK and CDNB strongly inhibit the outward

Table 1
Effects of various drugs on the floppase-mediated translocation of NBD-PS (or -PC), the flippase-mediated translocation of NBD-PS and the efflux of DNP-SG

Drug	Concentration (μmol l^{-1})	k/k_0		
		Flop of NBD-PS (-PC)	Flip of NBD-PS	Efflux of DNP-SG
None		1.00	1.00	1.00
Dipyridamole	20	0.05 (0.26)	1.26	0.53
Verapamil	50	0.10 (0.29)	1.17	0.91
Vinblastine	50	0.25	1.00	0.89
Colchicine	50	0.71	1.00	1.00
Quinidine	50	0.70	1.14	1.0
Daunomycin	15–30	0.05 (0.11)	1.05	
Staurosporine	20	0.05	1.12	
Vincristine	50	0.05	0.90	
Monensin	50	0.15 (0.47)	1.00	0.75
Rose bengal	20	0.71	0.66	0.57
Indomethacin	30	0.09 (0.22)	1.00	0.42
Genistein	150	0.32 (0.46)	1.00	0.44
Phloretin	250	0.28	1.07	0.27
Hexanoate	50,000	1.00	0.55	
Salicylate	20,000	0.43	0.21	
Benzbromarone	20	0.19	0.52	
Ethacrynic acid	50	0.05 (0.10)	1.14	0.77
CCCP	30	0.16	0.48	0.75
SDS	50	0.47	0.54	
Oligomycin	30	0.24 (0.38)	1.26	0.44
Triton X-100	150	0.33	1.00	0.68
CDNB[a]	1000	0.12 (0.05)	1.06	
TPCK[b]	200	0.05 (0.20)	1.56	0.73
TLCK[b]	500	0.05 (0.05)	1.55	0.88
PCMBS[c]	100	0.45	0.69	

The rate constants for the translocations mediated by the flippase and floppase as well as the DNP-SG efflux in the presence of drug (k) were related to those in their absence (k_0). The rate constants for the floppase-mediated component of translocation were obtained from the difference of the rate constants for the total flop and the leak flop (k_{leak}). The leak flop was determined after inhibition of the floppase by addition of 1 mmol l^{-1} vanadate or by pretreatment (15 min, 37°C) of cells with 0.8 mmol l^{-1} *N*-ethylmaleimide [7]. The mean values of k_{leak} for 18:1,6-NBD-PC and PS (at 37°C) are 0.0028 ± 0.0012 ($n = 12$; this work) and 0.0007 ± 0.0004 min^{-1} [28]. The effects of drugs on the floppase were routinely measured using NBD-PS as a substrate. In some cases, given in brackets, 18:1,6-NBD-PC was used. Data for the flip and flop of NBD-PS are mean values from resp. 2–12 and 2–7 experiments, data for the flop of NBD-PC and the DNP-SG efflux are mean values from 1–2 experiments.
[a,b,c] Pretreatment with the reagent for 30, 15 and 5 min, respectively at 37°C.

translocation of NBD-PS under these conditions, too. This proves that they really induce inhibition of floppase activity.

Floppase inhibition by the noncovalent drugs could also be confirmed using another phospholipid probe, NBD-PC containing an oleoyl and a 6-(NBD-amino)hexanoyl residue (18:1,6-NBD-PC). The outward flop kinetics of this probe, measured as usual in the absence of albumin (equilibrative flop), are not greatly affected by the passive flip rate of NBD-PC, which is very slow [7], and thereby end up in a low stationary level of NBD-PC in the inner leaflet (25% inside [7]). Presence during the flop of prostaglandin A1, ethacrynic acid (Fig. 4), indomethacin, genistein, monensin, benzbromarone, verapamil, daunomycin, dipyridamole, and oligomycin (Table 1) strongly suppresses the flop of NBD-PC. Similar results were obtained with another NBD-labeled PC containing a saturated myristoyl and a 12-(NBD-amino)lauroyl residue (14:0,12-NBD-PC) (data not shown). Flop-

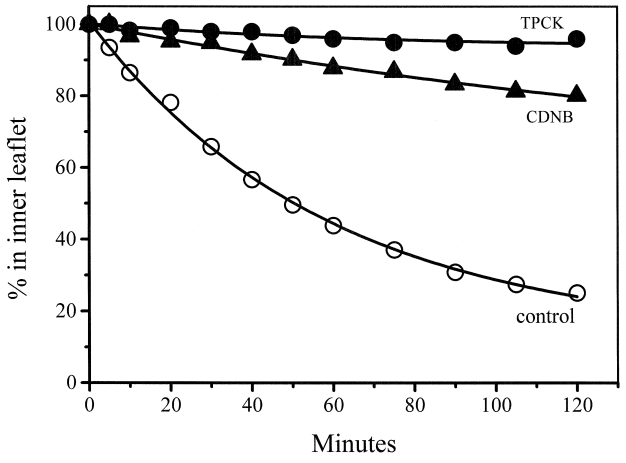

Fig. 3. Inhibition of the flop of NBD-PS by a pretreatment of cells with TPCK and CDNB. Flop in untreated cells (open circles) and in cells pretreated with TPCK (0.2 mmol l^{-1}, 15 min, 37°C) (solid circles) or CDNB (1 mmol l^{-1}, 30 min, 37°C) (triangles) was measured (1.5% hematocrit, 37°C) under zero-trans conditions, i.e., by continuously removing the probe from the outer leaflet by the presence of albumin (0.75 g dl^{-1}) in the medium during flop. Results from characteristic experiments out of a series of 3 for the same pretreatments.

pase inhibition is therefore responsible for the enhancement of the inward translocation rate and concomitant higher accumulation in the inner leaflet of NBD-PS after pretreatment of cells with TLCK, TPCK or CDNB and by various anionic and cationic drugs (Table 1).

In contrast to this strong and rather unselective inhibition of the floppase, the aminophospholipid flippase is not affected by the cationic drugs studied (Table 1). Among the anionic drugs tested, some are without effect on the flippase although being strong inhibitors of the floppase activity, while others have at least a moderate inhibitory effect. This is true for salicylate (79% at 20 mmol l^{-1}), CCCP (52% at 30 μmol l^{-1}), 2,4-dinitrophenol (43% at 2 mmol l^{-1}), benzbromarone (48% at 20 μmol l^{-1}), SDS (53% at 50 μmol l^{-1}) and hexanoate (45% at 50 mmol l^{-1}) (mean values for 2 experiments). The anionic phosphodiester, glycerophosphoserine, which has recently been claimed [31] to suppress the flippase in studies using an indirect test, proved to be ineffective. In our hands, the flippase-mediated translocation of NBD-PS measured by the albumin extraction procedure (see Section 2) was not inhibited by glycerophosphoserine at 0.5 mmol l^{-1} (data not shown). A short pretreatment (5 min) of cells with low concentrations (0.1 mmol l^{-1}) of the highly impermeable [32] sulfhydryl reagent, *p*-chloromercuribenzoate (PCMBS), inhibited the floppase by about 55% ($n = 2$; Table 1). This demonstrates the functional importance of an exofacial SH-group on the floppase. The same modification produced a lower inhibition of the flippase ($n = 2$; Table 1).

The different inhibitor sensitivities of the flip and flop process and also the different activation energies reported above make it very unlikely that both processes are mediated by the same protein.

Previously, we have presented evidence for a protein-mediated outward translocation of diacyl-PC synthesized from endogenous lysoPC by acylation with exogenous long-chain fatty acids at the inner surface of the erythrocyte membrane [4]. This translocation was fast, in contrast to the slow outward translocation of newly synthesized PE [4]. A similar substrate specificity has not been observed for the floppase-mediated translocation of NBD-phospholipids [6,7]. Moreover, the activation energy for the outward translocation of newly synthesized PC (30 kJ mol^{-1} [4]) was considerably lower than that of NBD-PC. Therefore, both processes are unlikely to be related.

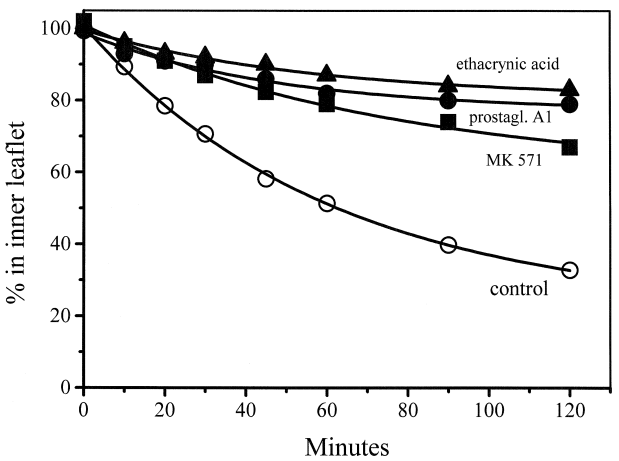

Fig. 4. Inhibition of the flop of 18:1,6-NBD-PC by addition of prostaglandin A1, ethacrynic acid and MK 571. Flop was measured at 37°C in the absence (open circles) and presence of 20 μmol l^{-1} prostaglandin A1 (solid circles), 100 μmol l^{-1} ethacrynic acid (up triangles) or 5 μmol l^{-1} MK 571 (squares). Results from characteristic experiments out of a series of 2–3 experiments at the same concentrations.

As proposed above, the inhibitory effect of the CDNB treatment on the floppase activity might be a consequence of the formation of DNP-SG. An inhibition of 50% was obtained after conjugation of about 500 μmol glutathione l^{-1} of packed red cells (data not shown). Glutathione conjugates are removed from erythrocytes by two active transport activities, one with high affinity and another one with low affinity for DNP-SG, also termed human multispecific organic anion transporters (hMOAT3 and hMOAT4). They have different substrate specificities and sensitivities towards inhibition by organic anions [14,16,33]. Both activities have the capacity to mediate the efflux of glutathione conjugates, mercapturates, organic anions, as well glucuronides [16,33]. There is growing evidence that the high affinity pump for glutathione conjugates is identical [17,34,35] with the multispecific transport protein associated with the development of multidrug resistance (MDR) and termed multidrug resistance protein (MRP), a 190-kDa protein belonging to the ATP-binding cassette (ABC) transporters [36–38]. The MRP1 isoform of the MRP family has been identified in tissue cells [34] as well as erythrocytes [17] while MRP2 is the isoform predominantly present in the hepatic canalicular membrane [39]. MRP has a very broad substrate specificity and not only transports anionic conjugates of drugs with glutathione, glucuronate and sulfate but also unmodified lipophilic cytotoxic anionic, neutral and cationic drugs [40–44]. If inhibition of the floppase-mediated translocation of NBD-phospholipids by CDNB would be due to the formation of DNP-SG, incubation of CDNB-pretreated cells should result in the removal of DNP-SG from the cells and thereby in a time-dependent loss of its inhibitory effect on translocation. This could indeed be demonstrated. Following a CDNB pretreatment of cells producing 50% inhibition of translocation, subsequent incubation of cells for 17 h at 37°C in the presence of energy supply (5 mmol l^{-1} inosine) decreased inhibition to below 10% (data not shown). Moreover, a direct inhibitory effect of DNP-SG on the floppase could be demonstrated in resealed ghosts containing various concentrations of DNP-SG, the glutathione conjugate of ethacrynic acid (EA-SG) or GSSG (Fig. 5). Data for the ratio of the rate constants in the absence (k_0) and presence (k) of drug from 3 to 5 experiments were plotted against the drug concentrations (Dixon plot) and the concentrations required to obtain 50% inhibition (IC_{50}) of the flop of NBD-labeled PS and PC were derived. The IC_{50} values were 100 μmol l^{-1} for DNP-SG, 70 μmol l^{-1} for EA-SG and 2 mmol l^{-1} for GSSG. Since GSSG is a substrate for the high affinity pump for glutathione conjugates [14,43] but is neither a substrate nor inhibitor for the low affinity pump [14], these results strongly suggest an involvement of the high affinity pump, i.e., MRP, in floppase activity. Further support for a role of MRP in floppase activity represents the dependence of both activities on GSH. Essentially no outward translocation of NBD-phopholipids was observed in the absence of GSH within the resealed ghosts (data not shown). GSH concentrations within the ghosts of 4–8 mmol l^{-1} are required to get flop kinetics similar to those for cells (cf. Figs. 4 and 5). Such a GSH-dependent transport has been reported for the transport of certain cationic drugs by MRP [41,44–47].

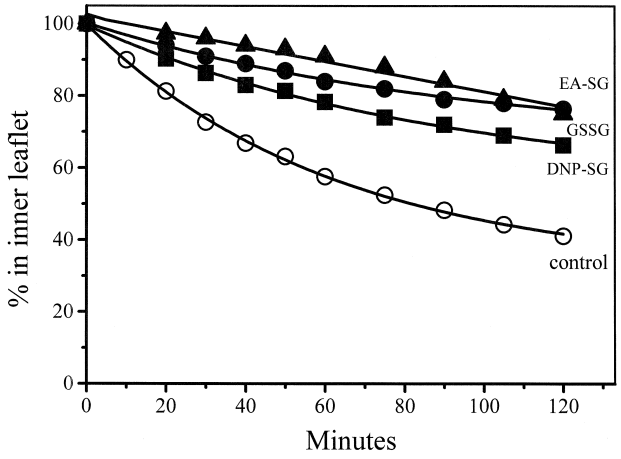

Fig. 5. Inhibition of the flop of 18:1,6-NBD-PC by trapping of DNP-SG, EA-SG or GSSG within resealed ghosts. After lysis of erythrocytes in a solution containing no additive (open circles), 300 μmol l^{-1} DNP-SG (squares), 150 μmol l^{-1} EA-SG (triangles) or 4 mmol l^{-1} GSSG (closed circles), resealed ghosts were prepared and flop measured at 37°C (see Section 2 for details). Results from characteristic experiments out of a series of 2 experiments at the same concentrations.

To exclude an involvement of the low affinity pump for glutathione conjugates in the floppase-mediated translocation of NBD-phospholipids, effects of inhibitors of the floppase on this pump were measured in erythrocytes. Under our experimental

conditions, the concentration of DNP-SG (500 μmol l^{-1} or higher) is well above the K_m value of the high affinity, low capacity transport system (3–4 μmol l^{-1} [14,33]) and its transport will take place predominantly via the low affinity, high capacity transport system ($K_m = 1$ mmol l^{-1} [14,33]). As shown in Table 1, the release of DNP-SG from the cells is only slightly inhibited after their pretreatment with TPCK or TLCK and by the presence during transport measurement of verapamil, vinblastine, monensin, ethacrynic acid or CCCP. In contrast, the floppase is strongly inhibited by these treatments (Table 1). Moreover, the temperature dependence for the low affinity transport system is considerably lower (61 kJ mol^{-1} [33]) than that of the NBD-phospholipid floppase (104 kJ mol^{-1} [7]). Finally, the low affinity pump is not affected by GSSG up to 12 mmol l^{-1} [14], whereas the floppase is strongly inhibited at 2–4 mmol l^{-1} (Fig. 5). It is concluded that the translocation system mediating NBD-phospholipid flop is certainly different from the low affinity pump for glutathione conjugates (MOAT4).

Further experiments aimed at collecting more evidence for a role of MRP in floppase activity and against such a role of another pump involved in multidrug resistance and member of the ABC family, the 170 kDa MDR1 P-glycoprotein [48,49]. P-glycoprotein is present in tumor cells and in the apical membrane of epithelial cells but has not been identified in the erythrocyte membrane as yet. In human cells, two classes of related P-glycoproteins expressed by the *MDR1* and *MDR2* gene were identified [22]. The MDR1 P-glycoprotein transports uncharged and cationic hydrophobic drugs [19–22,40,50–52] and was the first identified protein involved in multidrug resistance. Recently, MDR1 P-glycoprotein has been shown to transport NBD-labeled phospholipids, too [53–55]. On the other hand, the MDR2 P-glycoprotein does not transport drugs, but selectively transports PC in the canalicular membrane of hepatocytes [56,57]. It seemed therefore of interest to check whether floppase activity shares functional properties with P-glycoprotein.

This was done by studying the concentration-dependent effects, on the floppase-mediated translocation of NBD-phospholipids, of various drugs known to suppress MRP [18,40–43] and MDR1 P-glycoprotein [19–22] activity. Data for the ratio of the flop rate constants in the absence (k_0) and presence (k) of drug from 2 to 5 experiments were plotted against the drug concentrations and the concentrations required to obtain 50% inhibition (IC_{50}) of the flop of NBD-labeled PS and PC were derived. The IC_{50} values for verapamil, vinblastine and oligomycin were similar (Table 2) to those reported [22,40,58] for both the MRP protein and the MDR1 P-glycoprotein. On the other hand, the IC_{50} values for the floppase inhibition by anionic drugs such as genistein, indomethacin, CCCP, benzbromarone, prostaglandin A1 and ethacrynic acid are considerably lower (Table 2) than those reported for the MDR1 P-glycoprotein pump [40,58,59]. They were, however, similar to those established for the MRP pump [18,40,42,59,60]. Another potent, competitive inhibitor of MRP [17,43,61], the anionic leukotriene D_4 receptor antagonist MK 571 [24], which does not affect P-glycoprotein [61], strongly inhibited floppase activity (Fig. 4). At 5 μmol l^{-1} MK 571, the inhibition of the flop of 18:1,6-NBD-PC, 14:0,12-NBD-PC and NBD-PS were 65–70% in erythrocytes. The concentration of MK 571 required to obtain 50% inhibition of the floppase activity was 2.2 μmol l^{-1} for NBD-PS ($n = 3$),

Table 2
Drug concentrations required for 50% inhibition of the activities of floppase, MRP and MDR1

Drug	IC_{50} (μmol l^{-1})		
	Floppase[a]	MRP[b]	MDR1[b]
Oligomycin	5	1–2	1–2
Vinblastine	10	2–5	2–3
Verapamil	5–10	4–8	2–5
Ethacrynic acid	20	20–30	> 800
Indomethacin	5	10–20	> 800
Genistein	100	150–200	> 1,000
CCCP	10	10–30	> 200
Prostaglandin A1	5	3–5	> 100
Benzbromarone	5–10	5–10	> 500

[a]The concentrations of the drugs required to obtain 50% inhibition of the floppase-mediated outward translocation of NBD-labeled PS or 18:1,6-NBD-PC were derived from a Dixon plot of the ratio of the flip rate constants in the absence and presence of drug against drug concentration (2–5 experiments).
[b]Data taken from Ref. [40] represent the concentrations of the drugs required to obtain 50% inhibition of the efflux of calcein acetoxymethyl by MDR1 and MRP expressed in human tumor cells.

2.4 μmol l^{-1} for 14:0,12-NBD-PC ($n = 1$) and 2.7 μmol l^{-1} for 18:1,6-NBD-PC ($n = 2$) and thereby quite similar to that for MRP [17,43,61]. As shown above for other inhibitors of the floppase, the stationary distribution of NBD-PS following flip was also increased by addition of MK 571 (data not shown).

The close correlations between the inhibitory effects of various drugs on the floppase and MRP as well as the inhibition of the floppase by glutathione conjugates and GSSG trapped within resealed ghosts strongly suggest that the floppase mediating the translocation of NBD-phospholipids in the erythrocyte membrane is in fact the MRP pump. This function of MRP as a floppase for NBD-phospholipids may overlap with its physiological role of extruding toxic substances from the cell. Recently, the ether analog of PC, 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine, was identified by indirect methods as a substrate for several ABC transporters including MRP expressed in yeast [62]. The acceptance of a phospholipid by MRP may depend on the presence of an unphysiological hydrophobic group such as NBD or a short fatty acyl chain on the phospholipid molecule. It remains to be seen to what extent MRP (MOAT3) also accepts phospholipids with two long acyl chains. From the high membrane/water partition coefficients of the NBD-phospholipids (about 10^5; data not shown), a primary association of NBD-phospholipid with the floppase from the aqueous phase is unlikely. Therefore, MRP like MDR1 and MDR2 may act as a real floppase, i.e., as a transporter binding its substrate from the inner membrane leaflet and moving it to the outer leaflet. Depending on the lipid solubility, substrate binding could take place variably from either the membrane lipid bilayer or the aqueous medium. This would then correspond to the action of a classical exchanger of hydrophilic anions, band 3, that has been reported to operate as a translocase for membrane-associated anionic lipids [11–13]. Translocation of NBD-phospholipid via another mechanism, i.e., extraction of the probe from the inner membrane lipid leaflet and extrusion into the external aqueous medium, the so-called action as a vacuum cleaner [63,64], cannot be excluded, since the NBD-phospholipid released into the external aqueous medium would immediately redistribute into the outer membrane leaflet due to its high membrane/water partition coefficient.

Recently, MDR1 P-glycoprotein has been suggested to accept zwitterionic NBD-labeled phospholipids (PC and PE), but not the anionic NBD-PS [55]. This exclusion of PS corresponds to the established specificity of the MDR1 P-glycoprotein for neutral and cationic substrates [20–22]. On the other hand, MRP has been reported to transport neutral and cationic as well as anionic substrates [40–43]. Assuming that the floppase for NBD-phospholipids is really identical with MRP1, it is not surprising that it translocates mono- and di-anionic as well as zwitterionic NBD-phospholipids [6,7].

In studies on the kinetics and stationary distributions of NBD-labeled phospholipids as probes for endogenous phospholipids of cell membranes, the presence of MRP-mediated outward translocation should be considered.

Acknowledgements

We are indebted to Prof. B. Deuticke for his stimulating discussions and suggestions. We are grateful to Dr. A.W. Ford-Hutchinson of Merck Frosst Canada, for the gift of MK 571.

References

[1] P.F. Devaux, Annu. Rev. Biomol. Struct. 21 (1992) 417–439.
[2] C.F. Higgins, Cell 79 (1994) 93–395.
[3] X. Tang, M.S. Halleck, R.A. Schlegel, P. Williamson, Science 272 (1996) 1495–1497.
[4] C. Andrick, K. Bröring, B. Deuticke, C.W.M. Haest, Biochim. Biophys. Acta 1064 (1991) 235–241.
[5] M. Bitbol, P.F. Devaux, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 6783–6787.
[6] J. Connor, C.H. Pak, R.F.A. Zwaal, A.J. Schroit, J. Biol. Chem. 267 (1992) 19412–19417.
[7] M.V. Serra, D. Kamp, C.W.M. Haest, Biochim. Biophys. Acta 1282 (1996) 263–273.
[8] A. Zachowski, E. Favre, S. Cribier, P. Hervé, P.F. Devaux, Biochemistry 25 (1986) 2585–2590.
[9] F. Bassé, J.G. Stout, P.J. Sims, T. Wiedmer, J. Biol. Chem. 271 (1996) 17205–17210.
[10] Q. Zhou, J. Zhao, J.G. Stout, R.A. Luhm, T. Wiedmer, P.J. Sims, J. Biol. Chem. 272 (1997) 18240–18244.
[11] A. Vondenhof, A. Oslender, B. Deuticke, C.W.M. Haest, Biochemistry 33 (1994) 4517–4520.
[12] R. Ortwein, A. Oslender-Kohnen, B. Deuticke, Biochim. Biophys. Acta 1191 (1994) 317–323.

[13] A. Kleinhorst, A. Oslender, C. Haest, Biol. Chem. 377 (1996) S28.
[14] T.P.M. Akerboom, G. Bartosz, H. Sies, Biochim. Biophys. Acta 1103 (1992) 115–119.
[15] M. Heijn, R.P.J. Oude Elferink, P.L.M. Jansen, Am. J. Physiol. 262 (1992) C104–C110.
[16] M. Saxena, G.B. Henderson, Biochem. J. 320 (1996) 273–281.
[17] L. Pulaski, G. Jedlitschky, I. Leier, U. Buchholz, D. Keppler, Eur. J. Biochem. 241 (1996) 644–648.
[18] S. Paul, M.G. Belinsky, H. Shen, G.D. Kruh, Biochemistry 35 (1996) 13647–13655.
[19] M.M. Gottesman, I. Pastan, J. Biol. Chem. 263 (1988) 12163–12166.
[20] P.D. Roepe, Biochim. Biophys. Acta 1241 (1995) 385–405.
[21] C.R. Leveille-Webster, I.M. Arias, J. Membr. Biol. 143 (1995) 89–102.
[22] I. Bosch, J. Croop, Biochim. Biophys. Acta 1288 (1996) F37–F54.
[23] M. Saxena, G.G. Henderson, J. Biol. Chem. 270 (1995) 5312–5319.
[24] T.R. Jones, R. Zamboni, M. Belley, E. Champion, L. Charette, A.W. Ford-Hutchinson, R. Frenette, J-Y. Gauthier, S. Leger, P. Masson, C.S. McFarlane, H. Piechuta, J. Rokach, H. Williams, R.N. Young, Can. J. Physiol. Pharmacol. 67 (1989) 17–28.
[25] Y.A. Awasthi, G. Misra, D.K. Rassin, S.K. Srivastava, Br. J. Haematol. 55 (1983) 419–425.
[26] M. Coleau, P. Hervé, P. Fellman, P.F. Devaux, Chem. Phys. Lipids 57 (1991) 29–37.
[27] A.J. Verkleij, R.F.A. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn, L.L.M. Van Deenen, Biochim. Biophys. Acta 323 (1973) 178–193.
[28] C.W.M. Haest, D. Kamp, B. Deuticke, Biochim. Biophys. Acta 1325 (1997) 17–33.
[29] M. Bitbol, P.F. Devaux, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 6783–6787.
[30] G.E. Means, R.E. Feeney, Chemical Modification of Proteins, Holden-Day, San Francisco, 1971, pp. 105–110.
[31] D.J. Moore, R.H. Sills, R. Mendelsohn, Biochemistry 36 (1997) 660–664.
[32] P.A. Knauf, A. Rothstein, J. Gen. Physiol. 58 (1971) 211–223.
[33] G. Bartosz, H. Sies, T.P.M. Akerboom, Biochem. J. 292 (1993) 171–174.
[34] M.J. Flens, G.J.R. Zaman, P. van der Valk, M.A. Izquierdo, A.B. Schroeijers, G.L. Scheffer, P. van der Groep, M. de Haas, C.J.L.M. Meijer, R.J. Scheper, Am. J. Pathol. 148 (1996) 1237–1247.
[35] M. Heijn, J.H. Hooijberg, G.L. Scheffer, G. Szabó, H.V. Westerhoff, J. Lankelma, Biochim. Biophys. Acta 1326 (1997) 12–22.
[36] M.A. Barrand, T. Bagrij, S.Y. Neo, Gen. Pharmacol. 28 (1997) 639–645.
[37] S.P.C. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M.V. Duncan, R.G. Deeley, Science 258 (1992) 1650–1654.
[38] D. Lautier, Y. Canitrot, R.G. Deeley, S.P.C. Cole, Biochem. Pharmacol. 52 (1996) 967–977.
[39] D. Keppler, J. König, FASEB J. 11 (1997) 509–516.
[40] Z. Holló, L. Homolya, T. Hegedüs, B. Sarkadi, FEBS Lett. 383 (1996) 99–104.
[41] D.W. Loe, R.K. Stewart, T.E. Massey, R.G. Deeley, S.P. Cole, Mol. Pharmacol. 51 (1997) 1034–1041.
[42] S. Paul, L.M. Breuninger, G.D. Kruh, Biochemistry 35 (1996) 14003–14011.
[43] G. Jedlitschky, I. Leier, U. Buchholz, K. Barnouin, G. Kurz, D. Keppler, Cancer Res. 56 (1996) 988–994.
[44] D.W. Loe, K.C. Almquist, R.G. Deeley, S.P. Cole, J. Biol. Chem. 271 (1996) 9675–9682.
[45] G.J.R. Zaman, J. Lankelma, O. van Tellingen, J. Beijnen, H. Dekker, C. Paulusma, R.P.J. Oude Elferink, F. Baas, P. Borst, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7690–7694.
[46] C.H.M. Versantvoort, H.J. Broxterman, T. Bagrij, R.J. Scheper, P.R. Twentyman, Br. J. Cancer 72 (1995) 82–89.
[47] Y. Taguchi, A. Yoshida, Y. Takada, T. Komano, K. Ueda, FEBS Lett. 410 (1997) 11–14.
[48] C. Chen, J.E. Chin, K. Ueda, D. Clark, I. Pastan, M.M. Gottesman, I.B. Roninson, Cell 47 (1986) 381–389.
[49] P. Gros, J. Croop, D. Housman, Cell 47 (1986) 371–380.
[50] B. Sarkadi, M. Müllet, L. Homolya, Z. Holló, J. Seprodi, U.A. Germann, M.M. Gottesman, E.M. Price, R.C. Boucher, FASEB J. 8 (1994) 766–770.
[51] F.J. Sharom, X. Yu, J.W.K. Chu, C.A. Doige, Biochem. J. 308 (1995) 381–390.
[52] D. Keppler, I.M. Arias, FASEB J. 11 (1997) 15–18.
[53] A. Van Helvoort, A.J. Smith, H. Sprong, I. Fritzsche, A.H. Schinkel, P. Borst, G. Van Meer, Cell 87 (1996) 507–517.
[54] A. Van Helvoort, M.L. Giudici, M. Thielemans, G. Van Meer, J. Cell Sci. 110 (1997) 75–83.
[55] I. Bosch, K. Dunussi-Joannopoulos, R.L. Wu, S.T. Furlong, J. Croop, Biochemistry 36 (1997) 5685–5694.
[56] S. Ruetz, P. Gros, Cell 77 (1994) 1071–1081.
[57] R.P.J. Oude Elferink, G.N.J. Tytgat, A.K. Groen, FASEB J. 11 (1997) 19–28.
[58] J.M. Ford, W.N. Hait, Pharmacol. Rev. 42 (1990) 155–199.
[59] M.P. Draper, R.L. Martell, S.B. Levy, Br. J. Cancer 75 (1997) 810–815.
[60] C.H.M. Versantvoort, G.J. Schuurhuis, H.M. Pinedo, C.A. Eekman, C.M. Kuiper, J. Lankelma, H.J. Broxterman, Br. J. Cancer 68 (1993) 939–946.
[61] V. Gekeler, W. Ise, K.H. Sanders, W-R. Ulrich, J. Beck, Biochem. Biophys. Res. Commun. 208 (1995) 345–352.
[62] S. Ruetz, M. Brault, W. Dalton, P. Gros, Biochemistry 36 (1997) 8180–8188.
[63] M.M. Gottesman, Cancer Res. 53 (1993) 747–754.
[64] W.D. Stein, Physiol. Rev. 77 (1997) 545–590.