Multidrug resistance protein 1 regulates lipid asymmetry in erythrocyte membranes

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The role of multidrug resistance protein 1 (MRP1) in the maintenance of transbilayer lipid asymmetry in the erythrocyte membrane was investigated. The transbilayer distribution of endogenous phospholipids and [(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl (NBD)-labelled lipid analogues was compared in the absence and the presence of inhibitors of MRP1. At equilibrium the transbilayer distribution of the NBD analogues (in the absence of MRP1 inhibitors) was very similar to that of the endogenous lipids. Inhibition of MRP1 by verapamil or indomethacin resulted in a shift in the amount of probe that was internalized: approx. 50 % of NBD-labelled phosphatidylcholine (PtdCho) and 9 % of NBD-sphingomyelin (NBD-Spm) were no longer extractable by BSA in cells treated with inhibitor, in comparison with 25 % and 3 % for control cells respectively. To verify whether inhibition of MRP1 also affected the distribution of the endogenous phospholipids, phospholipase A2 and

sphingomyelinase were used to assess the amount of each of the various lipid classes present in the membrane outer leaflet. No shift in phospholipid distribution was observed after 5 h of incubation with verapamil or indomethacin. However, after 48 h of incubation with these inhibitors, significantly smaller amounts of PtdCho and Spm were present in the outer membrane leaflet. No appreciable change was observed in the distribution of phosphatidylethanolamine or phosphatidylserine. Decreased hydrolysis of PtdCho and Spm was not due to endovesicle formation, as revealed by electron microscopy. This is the first report to show that MRP1 has a role in the maintenance of the outwards orientation of endogenous choline-containing phospholipids in the erythrocyte membrane.

Key words: electron microscopy, lipid transport, 7-nitrobenz-2-oxa-1,3-diazole, phospholipase.

INTRODUCTION

The phospholipids of the plasma membrane of mammalian cells are asymmetrically distributed over both leaflets of the bilayer. Phosphatidylcholine (PtdCho) and sphingomyelin (Spm) are located predominantly in the outer leaflet, whereas the aminophospholipids phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) are found mainly in the inner leaflet (reviewed in [1]). An important contribution to the maintenance of lipid asymmetry comes from the activity of the aminophospholipid translocase, which specifically transports aminophospholipids from the outer leaflet to the inner leaflet at the expense of ATP [2,3]. In addition to this inwards transport, a non-specific outwards movement (flop) has been reported to be present in erythrocyte membranes [4–6]. This outwards movement, which was shown for both spin-labelled and NBD-labelled lipid probes, seems to be much slower than the inwards transport of PtdSer and PtdEtn by the aminophospholipid translocase. The putative so-called floppase, like the translocase, is inhibited at low temperature and by thiol reagents, and requires both ATP and GSSG for transport activity. Recent evidence has demonstrated that the floppase in erythrocytes is identical with multidrug resistance protein 1 (MRP1) [7,8], a member of the family of MRPs. MRP1 is an ATP-dependent membrane transport protein that, like the family of multidrug resistance (MDR) proteins, is responsible for expelling toxic and xenobiotic compounds from the cell [9]. Raggars et al. [10] demonstrated the transport

of sphingolipid analogues across the plasma membrane of LLC-PK1 kidney epithelial cells transfected with MRP1 cDNA. Like MRP1, MDR P-glycoproteins can also actively translocate different lipids across the membrane. Whereas transport by MDR3, which is most abundant in the bile-canalicular system, is restricted to PtdCho [11,12], other MDR P-glycoproteins transport a variety of lipids across the membrane [13,14].

Whereas most of these studies were restricted to transport of short-chain or fluorescently labelled lipid analogues, two groups of workers [13–16] have provided evidence that MDR P-glycoproteins fulfil a role in the transport of endogenous choline-containing phospholipids across the membrane. The aim of the present study was to obtain evidence for a role of MRP1 in the transbilayer distribution of endogenous phospholipids.

MATERIALS AND METHODS

Materials

1-Oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]hexanoyl-*sn*-sphingomyelin (C₆-NBD-Spm), 1-oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]hexanoyl-*sn*-glycero-3-phosphocholine (C₆-NBD-PtdCho) and 1-oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]hexanoyl-*sn*-glycero-3-phosphoserine (C₆-NBD-PtdSer), were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Verapamil, indomethacin, chlorpromazine and BSA were purchased from Sigma (St Louis, MO,

Abbreviations: C₆-NBD-, [(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-; MDR, multidrug resistance; MRP1, multidrug resistance protein 1; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; Spm, sphingomyelin.

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U.S.A.). Before the experiments, fresh stock solutions of the inhibitors were prepared: 100 mM verapamil in water; 1 M indomethacin in ethanol; 100 mM chlorpromazine in water. Phospholipase A₂ (EC 3.1.1.4) from *Naja naja* and from bee venom (*Apis mellifera*) and sphingomyelinase (EC 3.1.4.12) from *Staphylococcus aureus* were purified by the method of Zwaal et al. [17]. All other reagents were of the highest grade available commercially.

Isolation of erythrocytes

Whole blood was obtained from healthy volunteers by venipuncture, with acid citrate dextrose (ACD; 0.18 M glucose/0.08 M trisodium citrate/0.052 M citric acid) as anti-coagulant in a final ratio of 1:5 (v/v). Erythrocytes were collected by centrifugation and washed with a HEPES buffer (136 mM NaCl/2.7 mM KCl/2 mM MgCl₂/10 mM HEPES/0.2 mM EGTA/10 mM glucose, adjusted to pH 7.5). Cells were finally resuspended in the same buffer at a concentration of 5×10^8 erythrocytes/ml.

Inwards movement of C₆-NBD-phospholipids

Erythrocytes were incubated with 2 μ M C₆-NBD-probe, which corresponded to approx. 1% of the endogenous lipids. At zero time the inhibitors were added from their respective stock solutions and the incubation was followed at 37 °C. At intervals 40 μ l aliquots from the cell suspension were removed and diluted in 160 μ l of buffer with or without 1% (w/v) BSA. After extraction at room temperature for 1 min, cells were centrifuged at 12000 g and the pellets were solubilized in 2 ml of 1% (w/v) Triton X-100. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after extraction of the probe with BSA. The fluorescence intensities were measured at an excitation wavelength of 472 nm and emission wavelength of 534 nm. The integrity of the lipid probes was verified by TLC. No degradation of the probes was observed during the course of the experiments.

Treatment of erythrocytes with phospholipases and sphingomyelinase

Cells were incubated at 37 °C for 5 and 48 h in the presence or absence of 100 μ M verapamil or 250 μ M indomethacin. During the 48 h incubation, cells were washed and the buffer and inhibitor were replaced every 8 h. After incubation with inhibitor, erythrocytes were treated with phospholipases A₂ and sphingomyelinase to hydrolyse the lipids present in the outer leaflet, as described previously [18]. In brief, 5 ml of cell suspension was incubated with a mixture of 24 units of phospholipase A₂ from *N. naja* and 40 units of phospholipase A₂ from bee venom. After 30 min, 4 units of sphingomyelinase was added. The cells were incubated for another 2 h at 37 °C with occasional stirring to keep cells in suspension. During the incubation, haemolysis was measured routinely, and only when lysis remained below 5% were cells considered for further analysis. Hydrolysis was arrested by the addition of 15 mM EDTA, after which the cells were sedimented and analysed for phospholipid hydrolysis. Lipids were extracted and separated by two-dimensional TLC, followed by the determination of phosphorus as described by Verkleij et al. [18].

Transmission electron microscopy

After being washed, the cell suspension was centrifuged and the pellet was resuspended in 0.1 M phosphate buffer containing

2.5% (v/v) glutaraldehyde. After fixation, the cells were washed twice with 0.1 M phosphate buffer and post-fixed for 1 h with 1% (w/v) OsO₄ in 0.1 M phosphate buffer, dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections (approx. 70 nm) were cut on a Reichert–Jung ultracut microtome and collected on 75-mesh Formvar-coated copper grids; the grids were then contrasted with uranyl acetate in 50% (v/v) ethanol for 10 min, followed by staining for 5 min with lead citrate. The contrasted sections were dried in air and analysed in a Philips CM 10 microscope at 80 keV.

RESULTS

The addition of NBD-PtdCho to washed human erythrocytes results in a slow inwards transport of the probe, reaching equilibrium after approx. 6 h (Figure 1A, lower curve). Inspection of the uptake curve shows that, at equilibrium, 25% of the probe was present in the inner leaflet and 75% in the outer leaflet, closely reflecting the distribution of endogenous PtdCho in the erythrocyte membrane. Because NBD-lipid analogues have been

Figure 1 Inwards movement of fluorescent lipid analogues in human erythrocytes

(A) Inwards movement of C₆-NBD-PtdCho (NBD-PC) in the absence (●) and the presence of 100 μ M verapamil (■) or 250 μ M indomethacin (▲). Results are means $\pm$ S.D. for 17 independent experiments with verapamil and 9 independent experiments with indomethacin. (B) Inwards movement of C₆-NBD-Spm in the absence (●) and the presence (■) of 100 μ M verapamil. Results are means $\pm$ S.D. for four independent experiments. For reasons of clarity, results for Spm uptake in the presence of indomethacin are not shown, because they are indistinguishable from the results in the presence of verapamil.

Table 1 Phospholipid hydrolysis by the combined action of phospholipase A₂ and sphingomyelinase in erythrocytes incubated for 48 h in the absence or the presence of the MRP1 inhibitors verapamil or indomethacin

P values were obtained by comparison of the incubations with the use of paired Student's *t* tests. Values are means $\pm$ S.E.M. for 12 experiments in which verapamil (100 μ M) was used and 5 experiments with indomethacin (250 μ M) as the inhibitor of MRP1. The group of donors in the verapamil experiments was different from that in the indomethacin experiments, which is why the results for the two control groups are listed separately. Phospholipid hydrolysis was calculated by using PtdCho plus lysoPtdCho as internal standard. **P* < 0.01. Abbreviation: n.d., not detectable.

Lipid	Hydrolysis with verapamil (%)		Hydrolysis with indomethacin (%)	
	Control	MRP1-inhibited	Control	MRP1-inhibited
PtdCho	74 $\pm$ 2	67 $\pm$ 1*	73 $\pm$ 1	65 $\pm$ 2*
Spm	82 $\pm$ 1	77 $\pm$ 1*	78 $\pm$ 2	77 $\pm$ 2
PtdEtn	25 $\pm$ 1	26 $\pm$ 2	22 $\pm$ 2	20 $\pm$ 2
PtdSer	n.d.	n.d.	n.d.	n.d.

demonstrated to be a substrate for outwards transport by MRP1 [7,8], the equilibrium distribution of NBD-PtdCho presumably results from spontaneous diffusion across the bilayer and extrusion by MRP1. Consequently the inhibition of MRP1 was expected to result in a change in the equilibrium distribution of the probe. Indeed, as shown in Figure 1(A), incubation with 100 μ M verapamil, well above the IC₅₀ of 5–7 μ M [8], led to an increased uptake of NBD-PtdCho, levelling off at equimolar distribution. Similar results were obtained when 250 μ M indomethacin (IC₅₀ 44–55 μ M) was used to inhibit MRP1.

The inwards transport of NBD-Spm in the absence and the presence of verapamil is depicted in Figure 1(B). After 24 h, approx. 3% of NBD-Spm was no longer extractable by BSA in control cells incubated in the absence of inhibitor. This value was lower than expected on the basis of the distribution of endogenous Spm, of which approx. 20% is found in the inner leaflet of the erythrocyte membrane [18]. This might have been due to a low rate of transbilayer diffusion of this probe in comparison with NBD-PtdCho, so that equilibrium had not been fully attained even after incubation for 24 h. Although less pronounced than for NBD-PtdCho, an increased uptake of NBD-Spm to almost 10% was observed in erythrocytes incubated with verapamil or indomethacin. It should be noted that translocase-mediated uptake of NBD-PtdSer was not inhibited by verapamil or indomethacin (results not shown). In fact, a consistent but small increase in the uptake of NBD-PtdSer was found in the presence of these inhibitors. This confirms previous findings obtained with erythrocytes from MRP1 knock-out mice [8].

To find out whether the inhibition of MRP1 also affected the distribution of endogenous phospholipids, we used phospholipase A₂ in combination with sphingomyelinase to assess the phospholipid composition in the outer leaflet of the membrane. When erythrocytes that had been incubated for 5 h with verapamil or indomethacin were treated with phospholipases, no significant change in the pattern of hydrolysed lipids was observed in comparison with control erythrocytes (results not shown). However, this could be explained by the slow transbilayer movement of endogenous phospholipids in the erythrocyte membrane, which for PtdCho was estimated to have a half-time of between 3 and 26 h, depending on the acyl chain composition [19]. We therefore prolonged the incubation period with inhibitors to 48 h before analysing the lipids present in the outer leaflet. As shown in Table 1, the amount of PtdCho that could be

hydrolysed decreased from 74 $\pm$ 2% in control cells to 67 $\pm$ 1% in cells treated with verapamil, and from 73 $\pm$ 1% to 65 $\pm$ 2% in cells incubated with indomethacin. For Spm the hydrolysis was 82 $\pm$ 1% in control cells and 77 $\pm$ 1% in verapamil-treated cells, whereas the decrease in Spm hydrolysis was less obvious in cells treated with indomethacin. No significant change in hydrolysis of PtdEtn was found. Hydrolysis of PtdSer was not detectable in either control or inhibited cells.

These results seem to confirm earlier observations by Schrier et al. [20], who also found a decreased hydrolysis of PtdCho and Spm after the treatment of erythrocytes with amphipathic compounds such as primaquine and chlorpromazine. However, they demonstrated that at high concentrations these agents induce stomatocytosis and the subsequent formation of endovesicles that could explain the decreased hydrolysis of PtdCho and Spm. We therefore examined the morphology of the erythrocytes incubated with verapamil or indomethacin by electron microscopy. Figure 2 illustrates that no endovesicles were formed after incubation for 24 h with 100 μ M verapamil or 250 μ M indomethacin, whereas occasional endovesicle formation was observed after incubation for 48 h (Figures 2C–2F). As a positive control we used chlorpromazine at 200 μ M, which causes a massive production of endovesicles [20,21] (Figure 2B). In addition, in experiments to measure the inwards transport of NBD-lipid analogues under conditions inhibitory to MRP1, no appreciable endovesicle formation was found (Figures 2G and 2H). This indicates that, in MRP1-inhibited cells, the decreased accessibility of PtdCho and Spm to exogenously added phospholipases, and the increased amounts of fluorescent analogues of these lipids that were not extractable by BSA, were unrelated to the entrapment of lipid in endovesicle membranes.

DISCUSSION

The use of fluorescently labelled and spin-labelled lipid analogues has contributed significantly to a better understanding of the mechanisms that control the transbilayer distribution and movement of membrane lipids. At equilibrium, the distribution of most of the NBD- or spin-labelled analogues of the major phospholipid classes closely reflects the distribution of the endogenous lipids. Both types of lipid probe have been instrumental in the discovery and further characterization of the aminophospholipid translocase responsible for the fast and ATP-dependent inwards transport of aminophospholipids. In addition, these probes permitted the detection of a counter-transport, referred to as floppase activity, which is also ATP-dependent, but seemed to be non-specific with respect to the phospholipid polar headgroup [4–6]. Studies with MRP1 inhibitors [7] and MRP1 knock-out mice [8] have demonstrated that the outwards movement of NBD-labelled lipid analogues, observed in erythrocytes, is mediated by MRP1. Therefore the equilibrium distribution of NBD-labelled lipids might result from spontaneous diffusion across the bilayer, a fast inwards transport specific for aminophospholipids, and a much slower MRP1-mediated outwards transport that is non-specific. Blocking MRP1 causes a re-allocation of the lipid probes, which is most pronounced for NBD-PtdCho because this probe becomes equally distributed over both membrane leaflets. However, NBD-Spm does not seem to equilibrate at an equimolar distribution over both membrane leaflets. Although the apparently much lower rate of spontaneous diffusion of NBD-Spm would require a longer time for equilibration, it is clear from the curve in Figure 1(B) that an equal distribution over both leaflets is rather unlikely to be reached. This might suggest that, as well as MRP1,

Figure 2 Electron micrographs of human erythrocytes

(A) Control cells; (B) erythrocytes treated for 2 h with 200 μ M chlorpromazine (positive control); (C, D) erythrocytes treated with 100 μ M verapamil for 24 h (C) and 48 h (D); (E, F) erythrocytes treated with 250 μ M indomethacin for 24 h (E) and 48 h (F); (G, H) erythrocytes incubated with NBD-PtdCho for 5 h in the absence (G) and the presence (H) of verapamil. Arrows indicate endovesicles. Magnification is approx. 2800-fold.

another transporter is operative for NBD-Spm. Bettaieb et al. [15] and Bezombes et al. [16] have suggested that a P-glycoprotein, not defined further, has a role in the preferential outwards orientation of Spm in human leukaemia KG1a cells. Whether or not this protein is present in erythrocytes remains to be established. In addition, NBD-PtdSer was found to be transported to the outer membrane leaflet by MRP1, as shown previously [7,8]. Because inwards transport by the aminophospholipid translocase is much faster than the outwards movement by MRP1, more than 80 % of NBD-PtdSer is located in the inner leaflet at equilibrium. A minor but reproducible increased uptake of NBD-PtdSer was found in erythrocytes from MRP1 knock-out mice [8]. NBD-PtdEtn was not used in these studies because it does not always reflect the distribution of the endogenous PtdEtn [6,22]. An important question raised by the present observations with lipid analogues is whether MRP1 is also involved in the outwards transport of endogenous phospho-

lipids. This issue has also been raised with regard to the function of the related family of MDR proteins. van Helvoort et al. [13] have shown that, in addition to NBD-lipid analogues, short-acyl-chain analogues of PtdCho are also substrates for MDR1. With the exception of the transport of natural PtdCho by MDR3 in the bile-canalicular system [12] and the suggested role of P-glycoprotein in the distribution of endogenous Spm in leukaemic cells [15,16], no evidence for a function of MDR or MRP in the distribution of endogenous lipids has yet been presented. The present results demonstrate that prolonged inhibition of MRP1 in erythrocytes is associated with a shift in the distribution of endogenous PtdCho and Spm; the distributions of PtdEtn and PtdSer remained unchanged. The absence of PtdSer from the outer surface was also checked with the prothrombinase assay [8], an extremely sensitive method for detecting traces of PtdSer in the membrane outer leaflet. No change in prothrombinase activity of erythrocytes was found

after incubation for 48 h with verapamil or indomethacin. Schrier et al. [20], using erythrocytes treated with primaquine, vinblastine or chlorpromazine, also found a decreased hydrolysis of PtdCho and Spm by phospholipases without a change in the hydrolysis of PtdEtn or PtdSer. They demonstrated that the reorganization of membrane lipids was not due to a membrane-perturbing effect of these amphipathic compounds that would facilitate lipid migration across the bilayer. In contrast, they showed that the decreased hydrolysis of PtdCho and Spm was due to the entrapment of these lipids in endovesicles. According to the coupled bilayer principle, these endovesicles result from the intercalation of the amphipathic compounds in the membrane inner leaflet [20]. However, an electron-microscopic examination excludes endovesicle formation as a cause of decreased hydrolysis of PtdCho and Spm under our experimental conditions. The finding that the amount of PtdEtn hydrolysed remained unchanged supports this. Taken together, these findings suggest that the inhibition of MRP1 results in a net increase of phospholipid mass in the inner leaflet of the membrane that, unless corrected by an outwards movement of cholesterol, might eventually lead to the formation of endovesicles. The small increase in the limited number of endovesicles observed between the incubations for 24 and 48 h with verapamil or indomethacin might support this.

In summary, we have shown that the inhibition of MRP1 affects not only the distribution of phospholipid analogues but also that of the endogenous phospholipids. In the past, the preferential localization of choline-containing phospholipids in the outer leaflet of the membrane has often been explained to balance the inwards movement of PtdSer and PtdEtn by the activity of the aminophospholipid translocase (reviewed in [23]). However, it should be emphasized that verapamil or indomethacin, even after incubation for 48 h, does not have an inhibitory effect on the aminophospholipid translocase activity as measured with NBD-PtdSer. In addition, the outwards movement mediated by MRP1 after 48 h in the absence of inhibitors is not affected, indicating that sufficient amounts of ATP and GSSG are still present, both of which are cofactors for MRP1 activity [8]. Thus, by inhibiting MRP1 while leaving aminophospholipid translocase intact, the outwards orientation of choline-containing phospholipids decreases without compensation by aminophospholipids. We therefore conclude that MRP1 has an active role in the preferential orientation of PtdCho and Spm in the outer leaflet of the erythrocyte membrane, and that this orientation does not merely result from the active transport of aminophospholipids to the inner leaflet.

REFERENCES

- Zwaal, R. F. A. and Schroit, A. J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* **89**, 1121–1132
- Seigneuret, M. and Devaux, P. F. (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3751–3755
- Beleznyay, Z., Zachowski, A., Devaux, P. F., Navazo, M. P. and Ott, P. (1993) ATP-dependent aminophospholipid translocation in erythrocyte vesicles: stoichiometry of transport. *Biochemistry* **32**, 3146–3152
- Bitbol, M. and Devaux, P. F. (1988) Measurement of outward translocation of phospholipids across human erythrocyte membrane. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6783–6787
- Conner, J., Gillum, K. and Schroit, A. J. (1990) Maintenance of lipid asymmetry in red blood cells and ghosts: effect of divalent cations and serum albumin on the transbilayer distribution of phosphatidylserine. *Biochim. Biophys. Acta* **1025**, 82–86
- Connor, J., Pak, C. H., Zwaal, R. F. A. and Schroit, A. J. (1992) Bidirectional transbilayer movement of phospholipid analogs in human red blood cells. Evidence for an ATP-dependent and protein mediated process. *J. Biol. Chem.* **267**, 19412–19417
- Kamp, D. and Haest, C. W. M. (1998) Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane. *Biochim. Biophys. Acta* **1372**, 91–101
- Dekkers, D. W. C., Comfurius, P., Schroit, A. J., Bevers, E. M. and Zwaal, R. F. A. (1998) Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1). *Biochemistry* **37**, 14833–14837
- Keppler, D., Leier, I. and Jedlitschky, G. (1997) Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biol. Chem.* **378**, 787–791
- Raggers, R. J., van Helvoort, A., Evers, R. and van Meer, G. (1999) The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane. *J. Cell Sci.* **112**, 415–422
- Smit, J. J., Schinkel, A. H., Oude Elferink, R. P., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A., Ottenhoff, R., van der Lugt, N. M., van Roon, M. A. et al. (1993) Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75**, 451–462
- Smith, A. J., Timmermans-Herijgers, J. L. P. M., Roelofs, B., Wirtz, K. W. A., van Blitterswijk, W. J., Smit, J. J. M., Schinkel, A. H. and Borst, P. (1994) The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett.* **354**, 263–266
- van Helvoort, A., Smith, A. J., Sprong, H., Fritzsche, I., Schinkel, A. H., Borst, P. and van Meer, G. (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* **87**, 507–517
- van Helvoort, A., Giudici, M. L., Thielemans, M. and van Meer, G. (1997) Transport of sphingomyelin to the cell surface is inhibited by brefeldin A and in mitosis, where C6-NBD-sphingomyelin is translocated across the plasma membrane by a multidrug transporter activity. *J. Cell Sci.* **110**, 75–83
- Bettaieb, A., Record, M., Come, M. G., Bras, A. C., Chap, H., Laurent, G. and Jaffrezou, J. P. (1996) Opposite effects of tumor necrosis factor alpha on the sphingomyelin–ceramide pathway in two myeloid leukemia cell lines: role of traverse sphingomyelin distribution in the plasma membrane. *Blood* **88**, 1465–1472
- Bezombes, C., Maestre, N., Laurent, G., Levade, T., Bettaieb, A. and Jaffrezou, J. P. (1998) Restoration of TNF-alpha-induced ceramide generation and apoptosis in resistant human leukemia KG1a cells by the P-glycoprotein blocker PSC833. *FASEB J.* **12**, 101–109
- Zwaal, R. F. A., Roelofs, B., Comfurius, P. and van Deenen, L. L. M. (1975) Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. *Biochim. Biophys. Acta* **406**, 83–96
- Verkleij, A. J., Zwaal, R. F. A., Roelofs, B., Comfurius, P., Kastelijn, D. and van Deenen, L. L. M. (1973) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta* **323**, 178–193
- Middelkoop, E., Lubin, B. H., Op den Kamp, J. A. F. and Roelofs, B. (1986) Flip-flop rates of individual molecular species of phosphatidylcholine in the human red cell membrane. *Biochim. Biophys. Acta* **855**, 421–424
- Schrier, S. L., Chiu, D. T.-Y., Yee, M., Sizer, K. and Lubin, B. (1983) Alteration of membrane phospholipid bilayer organization in human erythrocytes during drug induced endocytosis. *J. Clin. Invest.* **72**, 1698–1705
- Isomaa, B., Hagerstrand, H. and Paatero, G. (1987) Shape transformations induced by amphiphiles in erythrocytes. *Biochim. Biophys. Acta* **899**, 93–103
- Morrot, G., Herve, P., Zachowski, A., Fellmann, P. and Devaux, P. F. (1989) Aminophospholipid translocase of human erythrocytes: phospholipid substrate specificity and effect of cholesterol. *Biochemistry* **28**, 3456–3462
- Williamson, P. and Schlegel, R. A. (1994) Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. *Mol. Membr. Biol.* **11**, 199–216