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Review

P-glycoprotein and 'lipid rafts': some ambiguous mutual relationships (floating on them, building them or meeting them by chance?)

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Abstract. P-glycoprotein (P-gp) is an active membrane transporter responsible for cell detoxification against numerous amphiphilic compounds, leading to multidrug resistance in tumor cells. It displays entangled connections with its membrane environment since it recognizes its substrates within the cytosolic leaflet and it also translocates some endogenous lipids to the exoplasmic leaflet. Regarding its relationships with membrane microdomains, 'lipid rafts', a literature analysis concludes that (i) P-gp also exists in rafts and non-raft membrane domains, depending on the cell considered, the experimental conditions and the method used to test it; (ii) cholesterol has a positive influence on P-gp function, and this may be a direct effect of the free cholesterol present in membrane or an indirect effect mediated by the cholesterol-enriched microdomains; (iii) when present in rafts, P-gp interacts with protein partners regulating its activity; (iv) P-gp is a lipid translocase that handles the raft-constituting lipids with particular efficiency, and it also influences membrane trafficking in the cell.

Keywords. P-glycoprotein, multidrug resistance, drug transport, lipid translocase, cholesterol, sphingolipids, lipid rafts, lipid traffic.

Introduction

The cell membrane must be considered as a cellular organelle that has the important role of regulating all kinds of exchanges (matter, energy, information and so on) between the cell interior and the external medium. To do that, it contains different proteins such as receptors and transporters whose activities depend on the surrounding lipids. Typically, lipids can influence membrane proteins by various means: at a geometric/steric level by determining the thickness or the curvature of the bilayer, at a dynamic level by determining the local microviscosity, at an electrochemical level by determining the local potential sensed by the charged residues and by direct interactions of low (e.g. 'lipid annulus') or high (e.g. 'structure lipid') affinity, and also indirectly by regulating protein-protein interactions. In other words, for a given membrane protein, lipids can be viewed as either non-specific and limited to a structural template, or specific and considered as functional metabolites. The current question about mutual relationships between P-glycoprotein (P-gp), the multidrug transporter, and the lipid microdomains, so-called 'rafts', is typically representative of this theme. This question is also relevant with regard to functional relationships with cholesterol, a membrane component exhibiting complex and ambivalent effects on both structural and functional characteristics of biological membranes. The aim of such a study is to deepen our understanding of P-gp function at the level of membrane enzymology as well as of cell physiology. Before report-

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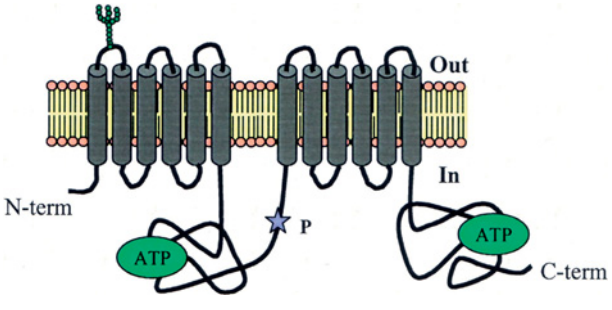
ing on the state of the art currently displayed in the literature, we will first present briefly each of the two 'partners' in question, P-gp and rafts.

P-gp as an active membrane transporter of a number of amphiphilic drugs and endogenous compounds

P-gp (ABC B1), a member of the large family of ABC (ATP-binding cassette) proteins, is a well-known transporter responsible for the multidrug resistance (MDR) phenotype in some cancer cells against a wide number of cytotoxic drugs [1]. P-gp is involved in clinical tumor resistance, either inherent or acquired, to various anticancer chemotherapy regimens, leading to therapeutic failure [2]. P-gp activity inducing MDR phenomena can be reproduced and studied *in vitro* in cultured cells of different origins [3]. P-gp is an active and multispecific membrane transporter, present in the plasma membrane and expelling cytotoxic drugs out of the cell at the expense of ATP hydrolysis; this leads to a reduced intracellular drug concentration and finally protects the cell from being killed [4]. P-gp is an integral membrane protein presenting a tandem structure composed of 12 transmembrane segments and two nucleotide binding domains, with a linker between the N- and C-terminal halves where phosphorylation sites, regulating its activity, are located (Scheme 1). The energetic coupling between MgATP hydrolysis and drug transport is carried out by an alternative working of the two nucleotide sites [5] and a rather large protein transconformation [6]. Remarkably, P-gp is able to transport both cytotoxic drugs and a huge number of molecules presenting a broad diversity of chemical structures, provided they are amphiphilic, neutral or cationic, and with a molecular weight ranging from ca. 250 to 1250 Da [7]. However, P-gp is a physiologically expressed protein, present in various healthy cells and tissues, such as cerebral capillary endothelial cells, intestinal mucosa, liver and kidney [8]. As a consequence, P-gp plays a key role in determining the pharmacokinetic properties of a number of pharmaceuticals, such as digestive absorption, cerebral disposition, and biliary and urinary elimination [9]. In addition, P-gp expression can be regulated by some of its transport substrates [8]. More recently, it appeared that P-gp is also able to handle some endogenous substrates, such as hydrophobic peptides [10] like cytokines [11], steroid metabolites [12] and even lipids [13, 14]. Although it is still unclear whether the translocated substrates are released in the exoplasmic leaflet or directly into the extracellular medium, a body of experimental evidence has established that P-gp transports its various substrates after binding them within the cytoplasmic leaflet of the plasma membrane [15, 16]. This may explain the high sensitivity of P-gp function on its hydrophobic environment, particularly on the state and composition of the surrounding membrane [17].



Scheme 1. Secondary structure prediction of P-gp, showing a tandem structure with a high homology between its N- and C-terminal halves, and presenting two transmembrane domains each composed of six helices (where transport substrates are recognized), two nucleotide binding domains (where MgATP is hydrolyzed), a set of different regulating phosphorylation sites ('P', star) and the glycosylated moiety branched on the first extracellular loop.

Membrane rafts as cholesterol-enriched lipid microdomains

Recent articles have extensively reviewed the structural properties and biological implications of lipid rafts in cell membrane. The orthodox presentation of membrane rafts consists of a model of compact and ordered association of cholesterol and sphingolipids, mainly sphingomyelin (SM) with long and saturated fatty acids bound to the sphingosine moiety, 'floating' in a more fluid membrane phase composed of glycerophospholipids having shorter and unsaturated acyl chains [18]. These lipid microdomains are characterized by a rather compact arrangement of the lipid molecules through specific H-bonds established between the cholesterol hydroxyl and the amide group presented by the sphingolipids, and they display an ordered hydrophobic phase in the membrane core due to the high saturation level of their acyl chains [19]. Their seminal chemical-physical property is a relative resistance to solubilization by some detergents under certain conditions (e.g. temperature); practically, this remains a current way of isolating these microdomains (although its validity is a matter of debate). Such membrane microdomains explain the molecular mechanism of lipid sorting in the cell, in particular concerning cholesterol and glycosphingolipids [20]. The biological relevancy of these microdomains also relies on the fact that they contain, either permanently or transiently, in some regulated manner, various proteins with diverse functions in cell physiology, such as signal transduction, membrane trafficking, lipid and protein sorting, and even receptors for some pathogens. In the important case of signal transduction, raft formation and/or coalescence provides the cell the opportunity of building transient platforms favoring specific interactions among the various partners of metabolic cascades, ensuring their efficiency [21, 22]. A typical protein present in some of these microdomains is caveolin (in cells where it is expressed), considered as a

marker of caveolae, which are partially invaginated cell membrane patches involved in endocytotic phenomena, and considered to be formed by the coalescence of preexisting rafts [23]. In addition, microdomains in the plasma membrane appear to be more or less specifically linked with actin constituting the underlying microfilament network [24].

However, in recent years, this model has begun to appear oversimplified. A first debate addresses the size of these lipid microdomains, which depends both on the method used for their analysis and on the membrane considered [25]. The apparent size of these microdomains, whatever the probe used (fluorescent marker, single particle tracking and so on), can also be the consequence of a heterogenous assembly of much smaller substructures giving the membrane a local property considered as a whole [26]. Possibly in this connection, reports show paradoxical responses to cholesterol depletion, leading to large-scale membrane domain formation and unexpected altered distribution of various raft markers [27]. Moreover, these microdomains, whatever their actual or apparent size, must be considered as dynamic structures of the cell membrane, with characteristic 'residency times' for their respective components, rather than stable and fixed domains. In addition, it has been proposed that the two leaflets of the microdomains present a certain asymmetry, with a rather more fluid cytosolic leaflet, but this remains still to be completely demonstrated [28]. Otherwise, the great diversity of lipid components in a cell membrane (as compared to a model membrane) makes it possible to understand that there can exist a certain heterogeneity of the formed microdomains. In particular, the exact lipid and protein composition of the microdomains clearly depends on the nature of the detergent used for assaying their solubilization resistance: 'Lubrol rafts' or 'Brij rafts' versus 'Triton rafts' are now reported [29–31]; the temperature at which the detergent is tested also appears to induce different microdomains to be isolated. In an effort to interpret these kinds of experimental data and reconcile them with MβCD sensitivity characteristics, it has been suggested that the boundaries of lipid microdomains may have a different lipid (and protein) composition compared with the core of the microdomains [19, 32]. This could have some implications for the coalescence of these

Table 1. Recapitulation of the various reported experimental findings of P-gp presence in membrane microdomains, depending on cell type, caveolin expression, cell lysis technique and eventual modulations.

Cell lines	CAV (+/–)	Microdomain assessment (detergent)	P-gp insolubility (low-density fraction)	Experimental modulation and effect on P-gp presence in rafts (+/–)	References
HT-29-MDR	+	TX100	40%		
MCF-AdrR	+	TX100	38%		[35]
BC-19	–	TX100	24%		
CEM/VLB5	+	TX100	main		[36]
Namalwa/MDR1		TX100	40%	Ab anti-CD19 (–)	[37]
L-MDR1	+	TX100	partial	MβCD (–)	[38]
MDR1-MDCK		TX100	main	MβCD (–)	[39]
Caco-2	+	carbonate	partial	DMβCD (–)	[40]
HepG2		carbonate	main	MβCD (–)	
CHO		carbonate	main	MβCD (–)	[41]
KB 8-5		carbonate	main	MβCD (–)	
NIH 3T3 MDR1		carbonate	partial	MβCD (–)	
K562/ADR	–	carbonate	no		[43]
CH^{R}C5	+	carbonate	partial	CSA or CCH (+)	[44]
BCEC	+	carbonate	partial		
NIH 3T3 MDR1		NP40/TX100	low/partial		[47]
HepG2	+	L-WX/TX100	main/no		[49]
MDCK	+	L-WX/TX100	main/no		
2780AD	–	Lubrol/TX100	main/part		[50]
CH(R)B30	+	Brij96/TX100	main/part		[51]
A549	+	OG/TX100	main/main		[52]

CAV, caveolin; TX100, Triton X100; NP40, Nonidet P40; L-WX, Lubrol WX; OG, octyl glucoside; Ab, antibody; CD/MβCD, (di-methyl-β-cyclodextrin; CSA, cyclosporin A; CCH, colchicine.

microdomains, for example in the key step of membrane signal transduction activation (known as 'capping' or 'patching') [33]. However, alternative models such as 'protein-lipid shell' [34] or 'induced-fit of raft heterogeneity' [31] have also been proposed.

Finally, it appears that answering the question of mutual relationships between P-gp and lipid microdomains is doubly difficult since both have been only partly described and understood, there are intrinsic theoretical and experimental subtilities, and the area is prone to rapidly evolve. Thus it is worth exploring the literature to establish the state of the art with respect to this question.

Influence of membrane structure and lipid composition on P-gp function

The first part of this literature overview will examine the various ways in which the membrane environment of P-gp, especially in relation to cholesterol and lipid microdomains, can influence its enzymologic functioning.

P-gp exists in detergent-resistant and low-density membrane microdomains

The first report of the presence of P-gp (24–40% of the total P-gp cellular content) in detergent-resistant membranes came from fractionation experiments after Triton X100 treatment of three different MDR cell lines, either expressing (HT-29 and MCF-7-AdrR) or not expressing (BC-19) caveolin [35]. Four subsequent reports (see Table 1) considered a selected MDR cell line (from CEM leukemia cells) where P-gp was found mainly in the detergent-resistant fraction [36], an *MDR1*-transfected B-lymphoma cell line where P-gp was found partly in the detergent-resistant fraction [37], and two MDR-transfected epithelial cell lines where P-gp was found to be distributed between the detergent-resistant and detergent-soluble fractions [38, 39]. In the three last cases, the proportions depended on the experimental conditions used (see below). Using a detergent-free, carbonate-based membrane fractionation of Caco-2 cells, it has been shown that P-gp was distributed between caveolar and non-caveolar fractions [40]. However, membrane fractionation of different cell lines expressing various amounts of P-gp showed that the lower the P-gp expression level, the higher the P-gp fraction in the low-density, cholesterol-enriched membranes [41]. Consistent with this data, we observed in the MDR cells DC-3F/ADX, a highly resistant cell line derived from a Chinese hamster lung fibroblast line selected with actinomycin D and overexpressing P-gp at a level of about 15% of total membrane proteins [42] that only a very minor fraction (in contrast to the major part of caveolin-1) of the high P-gp amount was actually found in the Triton X100-resistant membranes (Fig. 1). Conversely, the detergent-free membrane fractionation of MDR cells (K562/ADR cell line), devoid of caveolin, showed no P-gp in the low-density fraction [43]. Nevertheless, the P-gp localization in caveolar microdomains was detected on selected MDR cells (CH^RC5) as well as on spontaneously P-gp-expressing brain capillary endothelial cells. Coimmunoprecipitation of P-gp and caveolin was also shown in both cell types, indicating physical interaction between the two proteins [44]; this was later reported with both caveolin-1 and caveolin-2 as well [45]. Moreover, immunogold labelling of P-gp has been observed at the level of the caveolae in cultured astrocytes, and this localization was confirmed by immunoprecipitation with caveolin-1 [46]. Finally, the presence of P-gp in detergent-resistant membrane domains could be indirectly demonstrated by its association with cytoskeleton [47]. Indeed, P-gp has been shown to be colocalized and coimmunoprecipitated with ezrin, radixin and moesin, proteins known to interact with actin and to belong to raft microdomains [48].

However, other detergents have also been used to assay P-gp insolubility. In one such study, GFP-labelled P-gp was found in Lubrol WX-resistant but not in Triton X100-resistant (in contrast to caveolin) membrane fractions from both HepG2 cells and MDCK cells [49]. This is in agreement with data from an MDR cell devoid of caveolin-1 (2780AD cells), showing P-gp only partially present in the Triton X100-insoluble membrane domains but almost completely localized in the Lubrol-insoluble membrane domains [50]. Similarly, in ovary CH(R)B30 cells, P-gp was found entirely in Brij 96-insoluble membrane microdomains but only partially in the Triton X100-insoluble domains, in contrast to caveolin-1, with which P-gp did not colocalize by confocal fluorescence microscopy [51]. In addition, treatment by octyl glucoside of A549 MDR cells solubilized caveolin-1 but not P-gp, while they were both found in Triton-insoluble membranes, and no coimmunoprecipitation of P-gp/caveolin-1 was observed [52]. Solubilization experiments using CHAPS

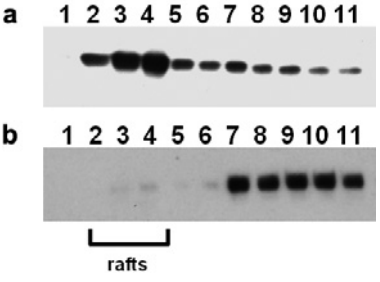


Figure 1. Triton X100 insolubility assay of P-gp-containing membranes. Cultured DC-3F/ADX cells were treated by 1% Triton X100 and homogenized at 4 °C, layered onto a 5–30% sucrose density gradient and centrifuged overnight. Eleven different fractions were harvested, washed, electrophoresed and finally submitted to immunodetection using C-13630 polyclonal antibodies against caveolin-1 (*a*) or monoclonal C494 antibodies against P-gp (*b*).

also indicated a high propensity for P-gp to remain in detergent-resistant membranes (see below).

P-gp is also present, and apparently functional for drug transport, in some internal membranes

The largely accepted dogma regarding cellular expression of P-gp states that it is present at the cell surface, and only there (in its maturated, functional form), in order to optimally exert its cellular function as an efflux pump. However, the literature shows recurrent reports of an intracellular location of P-gp. As a rather old example, the use of electron microscopy and immunofluorescence techniques have demonstrated the presence of P-gp also on the lumenal side of Golgi stack membranes and in small amounts in the endoplasmic reticulum (ER) of MDR KB-C4 cells [53]. This Golgi localization was subsequently confirmed by double-labelling and fluorescence microscopy experiments, which in addition showed that, in MDR MCF-7Dx cells, doxorubicin specifically accumulated in the Golgi apparatus, situated in the perinuclear region [54]. Moreover, P-gp has also been detected by immunogold labelling along the nuclear envelope in rat microglial cells [55], and in astrocytes [46]. Perinuclear localization of P-gp has also been recently reported in transfected HeLa cells, accompanied by doxorubicin sequestration in cytoplasmic vesicles harboring P-gp [56]. Similarly, sequestration of the two fluorescent MDR drugs, daunorubicin and Hoechst 33342, in P-gp-containing cytoplasmic vesicles was observed in highly resistant MDR cells (CH^RC5) by fluorescence imaging and immunoelectron microscopy [57]. This is consistent with subcellular anthracycline distribution, observed to shift from 'mainly nuclear' to 'mainly cytoplasmic' in different human squamous lung cancer MDR cells. This pattern correlated with P-gp expression level and increasing drug resistance [58], although the P-gp intracellular localization was not studied in these cells. Similarly, there was a correlation between the increasing degree of drug resistance in a series of selected MDR cells (from the Chinese hamster V79 line) and the amount of doxorubicin sequestered in cytoplasmic vesicles [59]. In these cells, while the *pgp1* gene expression positively correlated with drug resistance, the amount of P-gp in the plasma membrane reached a plateau at about 20%, suggesting a relatively higher participation of intracellular P-gp for the most resistant MDR cells, even if in this case an additional (e.g. increased drug metabolism) mechanism cannot be ruled out. This view is in agreement with electron microscopy observations, performed in our laboratory, of the highly resistant MDR cells DC-3F/ADX, which display a well developed internal membrane network (Fig. 2), although comparison with the parental sensitive DC-3F cells showed no constant or significant differences, but only a tendency to be more developed. However, in rat hepatocytes, no intracellular

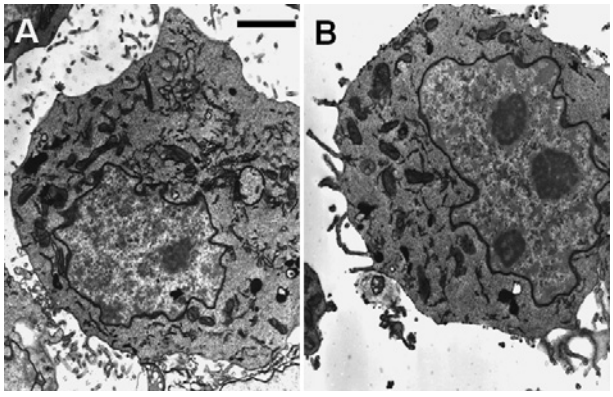


Figure 2. Electron micrograph of MDR cells, DC-3F/ADX (*a*) and their parental sensitive counterparts, DC-3F cells (*b*). The cultured cells were harvested, sedimented, fixed in glutaraldehyde and sectioned for electron microscopy; membranes were stained by osmium tetroxyde and lead citrate (according to Karnovski technique), and cells were photographed using an electron microscope (Philips CM12). Scale bar, 2 µM.

P-gp immunolabelling (using C219 antibodies) could be detected, in contrast to well-resolved immunofluorescent canalicular labelling [A. Cavelier and J.-M. Verbavatz, unpublished data]. Such an apparent discrepancy is likely to be attributed to the fact that in non-tumor cells, the amount of intracellular P-gp is generally much lower than in MDR cells.

More recently, the question has been revisited using P-gp/green fluorescent protein (GFP) fusion protein and a more dynamic point of view for cellular observations, in relation to membrane trafficking. Indeed, it has been shown that treatment of K562MDR cells by brefeldine A decreased surface P-gp level while leaving total P-gp level unaffected, whereas cycloheximide reduced total P-gp level while surface P-gp level was maintained, evidencing the dynamic interplay between cytosolic and surface pools of P-gp [60]. Furthermore, an MDR1-GFP fusion protein, expressed in transfected liver-derived polarized cells, was localized both in the canalicular membrane and in the subapical and Golgi regions, and it has ben shown that this fusion protein was directly transferred from the Golgi to the apical membrane involving membrane traffic regulated by phosphoinositide-3-kinase [61]. It addition, a recent report showed that neosynthesized P-gp, probed as an MDR1-GFP fusion protein, was directly transferred from the Golgi to the apical membrane of polarized HepG2 cells, and that this direct intracellular pathway was cholesterol-sensitive [49]. In this connection, it has been also observed in an MDR cell line (selected from an intestinal carcinoma) that P-gp undergoes constitutive endocytic trafficking, involving a pathway regulated by clathrin, adaptin and Rab5, and leading to about 30% of P-gp in the cell interior; furthermore, alteration of this trafficking induced decreased drug resistance [62]. Finally, in various *MDR1*-transfected cells, cryo-immunoelectron microsco-

py has shown that P-gp was predominantly intracellular, largely in Rab6-containing Golgi vesicles and Golgi cisternae, where it could participate in glycoceramide biosynthesis (see below) [63].

P-gp has enzymatic properties that tolerate, and even favor, a compact and ordered membrane environment

Since the membrane lipid phase provides the hydrophobic environment to (integral) membrane proteins, the local physico-chemical state of the bilayer obviously determines their functional activity. Among the membrane enzymes and transporters, P-gp is one of the most sensitive to its lipid environment, and this is likely linked to the fact that P-gp recognizes its transport substrates within the bilayer [16, 64]. Actually, many amphiphilic, membrane-perturbing agents, as well as exogenous incorporated lipids, display functional effects on P-gp activity. It was first reported that the membrane-rigidifying agent stearic acid and the cholesteryl esters hemisuccinate and phosphorylcholine inhibited efflux of rhodamine 123 (Rho123) out of MDR cells [65]. However, many other molecules known to fluidize the cell membrane were also observed to have an inhibiting effect on P-gp function, as assayed either by cell efflux or vesicle uptake of drugs, or by ATPase activity: linoleic acid [65], the octanoate derivative A_2C [66], benzyl alcohol [65–68], chloroform and diethyl ether [68], mild detergents and surfactants [67, 68]. More generally, the influence of lipid composition in the environment of P-gp on its activity was evidenced after reconstitution of P-gp activity in different lipid mixtures, leading to various ATPase drug-induced stimulation profiles [69], and this could be more directly studied by reconstitution of (purified) P-gp in proteoliposomes made of defined lipids. Sharom and co-workers have shown successively that (i) when P-gp is reconstituted in gel phase lipid, the Michaelis constant for ATP is lower than in liquid-crystalline lipid, with a lower activation energy for ATP hydrolysis just below the melting transition [70] (ii) binding of three representative P-gp substrates (vinblastine, daunorubicin and verapamil) presents a 2- to 4-fold higher affinity in gel phase lipid than in liquid-crystalline lipid [71]; and (iii) the transport rate of tetramethylrosamine, a P-gp fluorescent substrate, is higher in the rigid gel phase than in the fluid liquid crystalline phase [72]. However, in natural cell membranes, lipid heterogeneity likely results in more complicated relationships between P-gp and its membrane environment.

P-gp function is sensitive to membrane cholesterol depletion/repletion, and perhaps also to glycosphingolipids

Beside the other endogenous membrane components, cholesterol has received special attention aimed at testing for possible influence on P-gp function, assayed by different means. With respect to drug binding, it was first reported that the cholesterol content of purified P-gp-containing proteoliposomes influenced the amount of azidopine (a photoactivatable dihydropyridine) able to specifically photolabel P-gp according to a bell-shaped curve, with a maximum at a weight ratio of 20% to phosphatidylcholine (PC) and virtually no effect at 40% [73]. This is consistent with the absence of effect of adding 45% mol/mol cholesterol to purified P-gp, reconstituted in proteoliposomes of various lipid compositions, on the binding potency of nicardipine (another dihydropyridine) or of XR9576, an anthranilic acid derivative [74]. However, using fluorescent-labelled purified and reconstituted P-gp, and a quenching assay, it has been shown that inclusion of 20% w/w cholesterol to the proteoliposomes decreased by 10 times vinblastine affinity without any alteration of the binding of either verapamil or daunorubicin [71]. In contrast, cholesterol depletion (using MβCD) from the cell membrane of Caco-2 cells induced a decrease in the specific binding of saquinavir [75].

Analysis of the effect of cholesterol on P-gp ATPase activity provided more consistent results. On the one hand, incorporation of up to 30% cholesterol in purified P-gp-containing PC:PE-based proteoliposomes induced a dose-dependent increase in the basal (i.e. in the absence of added drug) ATPase activity of P-gp, with a concomitent decrease in the ability of verapamil to stimulate this activity [76]. Similarly, adding 45% mol/mol cholesterol to purified P-gp, reconstituted in proteoliposomes of various lipid compositions, induced a tendency to increase the basal ATPase activity of P-gp with no effect on the stimulation of this activity by nicardipine [74]. When tested on microsomes prepared from *MDR1*-transfected cells, cholesterol has been shown to induce stimulation of P-gp ATPase activity [77]. On the other hand, P-gp ATPase activity measurement, performed on plasma membranes prepared after cholesterol depletion (by approximately 50%) in MDR CEM cells, showed roughly half-inhibition compared with control membranes, and this effect was reversed by cholesterol repletion of the cells [78]. Furthermore, in membrane vesicles prepared either from selected MDR cells (DC-3F/ADX) or from *MDR1* gene-transfected insect cells, the basal P-gp ATPase activity was vanishing when they were progressively cholesterol depleted, using either digitonin or MβCD, and this was reversed by reincorporating cholesterol; after cholesterol depletion, verapamil or progesterone were still able to stimulate P-gp ATPase, but to a level somewhat lower than that reached with native membranes [79]. The different effect of cholesterol when modulating the basal or drug-stimulated ATPase activity of P-gp makes it rather unlikely that cholesterol influences P-gp function by indirect means, such as membrane fluidity changes.

As for drug transport by P-gp, determined either by direct measurements or by cytotoxicity assays, different research efforts have evidenced an influence of cholesterol in the cell membrane. First of all, it should be mentioned that membrane cholesterol plays a noticeable role in the passive uptake of amphiphilic drugs into cells which must be distinguished from an actual, direct effect on P-gp transport function. For example, cholesterol deprivation (over the long term, which could induce P-gp expression regulation, see below) in the cell culture medium caused reduced doxorubicin uptake into an MDR human ovarian cancer cell line, and this was even more marked in the sensitive parental cell line [80]. In contrast, cell cholesterol depletion/repletion induced a similar effect on vincristine uptake in sensitive and MDR murine leukemic cells, the rate of drug uptake being inversely correlated with the molar ratio of cholesterol to phospholipids [81]. Moreover, cholesterol addition to cholesterol-depleted ('repletion') or untreated ('saturation') human lymphocytes and monocytes led to a marked decrease in Rho123 cellular uptake [82]. In a somewhat comparable manner, addition of cholesterol to phosphatidylinositol-containing liposomes counterbalanced their increasing effect on vinblastine uptake by the treated MDR CEM cells, while it was ineffective alone and had only a moderate effect on the sensitive parental cells [83]. In addition, treatment by MβCD of MDR and sensitive parental cells (HL-60 or MCF-7) induced an increased cytotoxicity of doxorubicin in all cell lines, but in a slightly more clear-cut manner for the MDR cells [84]. However, addition of cholesterol was observed to inhibit daunorubicin efflux out of *MDR1*-transfected cells but not in control cells, whereas it was without effect in Rho123 cell uptake [77]. Recently, it was reported that MβCD-induced cholesterol depletion of two related MDR cell lines provoked a clear increase of daunomycin intracellular retention, linked to chemosensitization of these cells, a phenomenon only partially reversed by cholesterol repletion of the cells, and which was not observed on their parental sensitive counterparts [78]. Finally, cholesterol depletion and repletion of MDR K562/ADR cells showed a linear direct correlation between the cellular cholesterol content and the active efflux activity of P-gp transporting pirarubicin [43]; interestingly, treatment of these cells by cholesterol oxidase, leading to subtotal oxidation of the membrane cholesterol, did not induce any inhibition of P-gp transport function, which can be taken as indicating that P-gp does not reside in raft domains and that cholestenone displays the same capability as cholesterol to sustain P-gp activity. However, from these transport experiments, and even in this last case, it is difficult to unambiguously distinguish between a direct, specific cholesterol effect and an indirect, membrane-mediated effect on P-gp.

Concerning the other important constituents of rafts, glycosphingolipids, there are some reports about testing their involvement either directly or indirectly. The direct functional effects of some rather soluble ceramide derivatives (with a C_6 short-chain) added exogenously were tested on P-gp transport activity, assayed by Rho123 efflux and doxorubicin cytotoxicity on ovarian 2780AD cells. This work showed inhibition by the neutral hexanoylglucosylceramide, but not by the other glycolipids tested, showing the importance of the headgroup [85]. However, indirect manipulations of the cells using PDMP, a GlcCer biosynthesis inhibitor, or sphingomyelinase did not induce any change in P-gp efflux function. Conversely, PDMP induced moderate inhibition of P-gp-mediated Rho123 efflux in HepG2 cells [86]. In contrast, targeting of GlcCer synthase expression by either transfection of its gene or of antisense oligonucleotide gave more clear-cut results. In situ GlcCer synthesis activation induced resistance to doxorubicin cytotoxicity in both MCF-7 parental sensitive [87] and MDR cell lines [88], whereas in situ GlcCer synthesis inhibition [89] induced sensitization to doxorubicin (and other MDR drugs) cytotoxicity much more marked in the MDR cells than in the sensitive cell line [90]; sensitivity against non-MDR cytotoxic drugs as well as P-gp-mediated Rho123 efflux were unchanged [88]. Finally, PDMP treatment of MDR myeloid leukemia cells induced increases in both Rho123 retention and chemosensitization, and among all the glycoceramides tested, this inhibiting effect of P-gp function was reversed only by the gangliosides GM3 and GD3 [91].

Membrane microdomains influence ATPase activity and the multidrug transport function of P-gp

The dependence of P-gp function on its insertion in lipid microdomains was directly investigated in some work. First, preparation from MDR cells of a P-gp-containing detergent-resistant membrane fraction showed that it displayed ATPase activity activatable by verapamil and reversibly sensitive to cholesterol depletion; interestingly, this contrasted with the smaller population of P-gp present in the detergent-sensitive membrane fraction, which displayed ATPase activity increased by extra cholesterol added and inhibited by verapamil [36]. Similarly, P-gp contained in membrane fractions prepared by differential extraction in cold Triton X100 showed ATPase activity qualitatively similar to that of crude membranes, and P-gp was clearly enriched in this raft fraction with respect to the crude membranes [92]. In this context, it is conceivable (although not mentioned by the authors) that the preparation of partially purified P-gp by using CHAPS, a detergent known to be rather weakly solubilizing and cholesterol preserving [93], for a selective differential extraction would have led to somewhat similar, raft-like enriched membrane fractions (as reported for another membrane protein [94]), which was further used for detailed analysis of P-gp ATPase activity [95]. Actually, treatment

of P-gp-containing membrane vesicles, prepared from the highly resistant DC-3F/ADX cell line, by CHAPS under optimized conditions led to only 30% P-gp solubilization (i.e. in micellar form found in a 100000 g supernatent) compared with 70% of total membrane proteins solubilized, while other detergents tested in parallel (such as $C_{12}E_8$, octyl glucoside or deoxycholate) led to about double the amount of P-gp solubilized but only to slightly more total membrane proteins solubilized [64]. In this case, the two P-gp-containing fractions, solubilized and non-solubilized, obtained after CHAPS treatment exhibited comparable basal ATPase activity. The non-solubilized membranes were still able to display verapamil-stimulated ATPase activity comparable to native membranes, but P-gp included in mixed micelles lost all capability to be stimulated by verapamil. When P-gp-containing membranes are submitted to increasing CHAPS concentrations, preservation of the basal ATPase activity of P-gp included in mixed micelles is illustrated by a steep increase of this ATPase activity accompanying the solubilization threshold (control curve in Fig. 3, and [64]). When the P-gp environment is depleted (by MβCD or saponin treatment) or devoid of cholesterol (membranes from insect cells), this steep increase is virtually absent (Fig. 3). This is a further indication of the importance of cholesterol in the P-gp environment for its basal ATPase activity, in micelles as well as in membranes, besides the possible direct involvement of rafts.

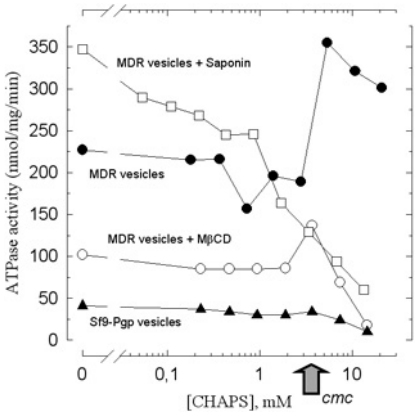


Figure 3. Effect of increasing CHAPS concentrations on the basal ATPase activity of P-gp. P-gp is present in membranes prepared from DC-3F/ADX cells, in the absence (closed circles) or presence of 10 mM MβCD (open circles) or of 50 µg/ml saponin (open squares), or prepared from *MDR1*-transfected Sf9 insect cells (closed triangles). MβCD subtotally extracts cholesterol from the treated membranes, saponin in situ sequesters cholesterol when permeabilizing the treated membranes, and Sf9 insect cell membranes contain a very low amount of endogenous cholesterol, as reported in [79]. ATPase activity is measured at 37 °C by the continuous monitoring of NADH absorbance in a thermoregulated and well-stirred spectrophotometer cuvette using a coupled enzyme assay composed of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase, and in the presence of the ion pump inhibitors azide, ouabain and EGTA, allowing successive addings of CHAPS to the P-gp-containing membrane suspension, as described in [64]; cmc, critical micelle concentration (3–5 mM).

Other research has addressed the question of the relationship between membrane microdomains and P-gp through studies at the cellular level, generally using cholesterol depletion and/or repletion procedures. Partial cholesterol depletion obtained by treatment of Caco-2 cells in a monolayer by DMβCD induced inhibition of P-gp-mediated Rho123 transport, and this was accompanied by partial release of P-gp into the apical side medium, mainly from the cholesterol-rich fraction, while caveolin and flotillin-1 remained unaffected [40]. This observation is consistent with the above-mentioned data showing P-gp in microdomains of 'intermediate solubility'; however, it is difficult to affirm a direct relationship between the membrane cholesterol level and the presence of P-gp in membrane microdomains. Similarly, MβCD treatment of *mdr1*-transfected MDCK cells induced a shift of P-gp from raft fractions to higher-density fractions; interestingly, treatment of the cells by the GlcCer synthase inhibitor PDMP gave the same effect (see below) [39]. Also, on P-gp-expressing epithelial cells, cholesterol depletion induced both a shift in P-gp localization from detergent-resistant to detergent-soluble membranes and P-gp-dependent increased cellular uptake of bodipy-verapamil (a fluorescent P-gp substrate), cholesterol repletion or saturation giving the opposite effects [38]. However, these data may be due either to a supportive effect of cholesterol-rich membrane microdomains to drug transport P-gp function or to a direct positive role of cholesterol on this P-gp function as well, independent of P-gp presence in the rafts. Actually, comparison of various MDR cells from different species showed different responses to MβCD-induced cholesterol depletion, also depending on the drug tested for transport (Tc-Sestamibi or daunomycin), and, as a whole, the drug transport function of P-gp appeared more dependent on cell cholesterol content than on localization in low-density membrane fractions [41]. Otherwise, overexpression of caveolin-1 induces both a decrease in plasma membrane cholesterol (more marked in the MDR cell line than in the sensitive counterpart) and P-gp transport activity and an increase in membrane fluidity similar to the effects of cell treatment by cyclodextrins, suggesting an indirect role of caveolin-1 [96], although the correlation with membrane fluidity cannot be ascertained to be the cause of P-gp inhibition.

However, there are some reports of direct correlation between P-gp function and its association to lipid raft components. For example, mutations in the caveolin-binding motif present in P-gp both reduced interaction of P-gp with caveolin-1 and increased the transport activity of P-gp [45]. Along the same lines, treatment of P-gp-expressing cells by antisense oligonucleotides against ezrin, radixin and moesin inhibited both P-gp-mediated drug efflux and P-gp association to actin, accompanied by a re-

distribution of P-gp at the cell surface [48]. Similarly, treatment of MDR cells with a monoclonal antibody against CD19, a B-cell surface receptor distributed between detergent-insoluble and soluble membrane fractions, induced disruption of the interaction between P-gp and CD19 and release of P-gp out of the raft fractions (and an increased association of CD19 to rafts), accompanied by chemosensitization of the cells [37]. Nevertheless, in these reports, specific interactions of P-gp with certain protein partners present in membrane rafts may have a functional importance priming its own presence in these lipid microdomains. Moreover, although alterations of P-gp function are obtained by cellular cholesterol manipulations which perturb raft domains, and are accompanied by P-gp release out of these rafts (when tested), this does not necessarily involve raft as the (sole) functional localization of P-gp, since cholesterol may also be a direct or indirect causal agent for modulating P-gp function on its own. Thus, at this point, the question remains open whether P-gp exhibits neighborhood relationships with cholesterol-enriched microdomains in the cell membrane within a simple non-specific context with surrounding lipids, or whether it takes advantage of a specific role of cholesterol that can also be encountered outside the rafts.

P-gp expression (level and location) is regulated by cellular cholesterol and coregulated with caveolin expression

Membrane composition, especially regarding cholesterol, has an influence not only on P-gp function but also on P-gp expression level. In particular, cellular cholesterol loading and depletion, using low-density lipoprotein (LPL) and high-density lipoprotein (HDL), respectively, induces up- and downregulation, respectively, of P-gp expression in human macrophages; however, the cellular cholesterol level can also modify the expression level of a number of other human ABC transporters [97]. Similarly, there is a positive correlation between the content of cholesteryl esters in normal or atherosclerotic human artery walls and *MDR1* gene expression [98]. The same group further showed a positive correlation between MDR1 and ACAT expression (and proliferation propensity), but a negative correlation with caveolin-1 expression [99].

More specifically, caveolin-1 overexpression after transfection in MDR cells (containing low levels of endogenous caveolin-1) induced almost complete downregulation of P-gp expression, leading to reversion of the drug resistance [100, 101]. Conversely, it has been simultaneously reported by two research teams that there is a coregulation of the expression of P-gp and caveolin, since caveolin-1 (and caveolin-2 in one case) was overexpressed in different selected MDR cells [35, 102]. Moreover, in different clinical groups of normal and leukemic bone marrow cell samples, there was a positive correlation between MDR1 and caveolin-1 gene expression [103]. However, this co-regulation is not a strict characteristic because (i) a pair of MDR and parental sensitive cell lines may not show any difference in caveolin expression level [44, 52]; (ii) caveolin is not overexpressed in one cell line stably transfected by P-gp [35], and more generally there are various cell types devoid of caveolin expression that express P-gp very well; (iii) caveolin was overexpressed in drug-resistant cells expressing no P-gp [102], and more generally caveolin expression level can be altered by various cell stimuli and experimental conditions [104]. Nevertheless, it is interesting that such a co-regulation should be posited in connection with the existence of a specific and functionally relevant interaction between P-gp and caveolin, as mentioned above [45]. This points out the possible functional importance of the cellular localization of these two proteins in regulation of their mutual expression. As an illustration, we observed that in the highly resistant MDR cell line DC-3F/ADX, caveolin-1 is moderately overexpressed (about 2 times) in total cell extracts compared with the parental sensitive cell line (DC-3F), whereas, remarkably, the caveolin-1 level is dramatically increased (about 10 times) in the membrane fraction from the MDR cells over the control cells (Fig. 4). Also noticeable in this context is the report of increased expression of both caveolin and P-gp in caveolar domains (whereas their cellular expression levels were

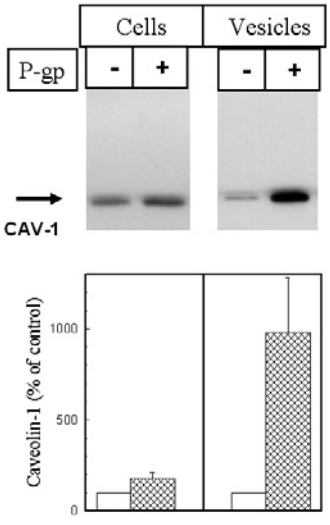


Figure 4. Caveolin-1 quantitation in total cell extracts and in membrane fractions from MDR cells. Total protein samples (0.5 µg) from DC-3F or DC-3F/ADX cells (left panel; n = 2) and membrane protein samples (0.5 µg) from vesicles prepared from these two cell lines (right panel; n = 3) were subjected to SDS-PAGE polyacrylamide gel electrophoresis and caveolin-1 was immunodetected using the C-13630 polyclonal antibody. The bands were quantified by densitometry. Error bars represent the standard deviation of the ratio of MDR to control.

unchanged) induced by culturing MDR cells in the presence of P-gp transport substrates, the MDR drug colchicine or the MDR reversing agent cyclosporin A [44]. This is well in line with the proposal of Liscovitch and Lavie according to which intracellular MDR drugs can be pulled into caveolin-containing cytosolic platforms, thanks to their hydrophobicity, and then take advantage of the intracellular traffic of these microparticles to be presented to P-gp within the plasma membrane and eventually expelled out of the cell [105].

As an intermediate conclusion, it could be inferred from the cholesterol-dependence of the P-gp cellular expression level that P-gp possibly behaves like a stress-inducible protein, whose physiological function would involve more or less direct action on cholesterol or some functionally related lipid membrane components. Consistent with this possibility is the observed repercussion of P-gp presence/distribution on cellular caveolin, taking into account the importance of this protein in cell cholesterol metabolism.

Influence of P-gp on lipid translocation, membrane traffic and membrane-mediated cellular events

In this part, we will focus on the different consequences, described in the literature, of P-gp expression on the cell membranes, both at a structural and at a functional level, which can be more or less directly related to membrane microdomains.

P-gp appears to act as a lipid translocase

The first report of lipid translocase activity for P-gp was established on *MDR1*-transfected epithelial cells, where, compared with control cells, an increased exchange of labelled short-chain phospholipids from the cell interior (where they were synthesized) to albumin, used as a lipid acceptor in the external medium, was evidenced under conditions that precluded any participation of exocytosis processes [13]. This outward intramembrane lipid translocation ('floppase' activity) was observed for various polar headgroups, PC, PE and GlcCer, consistent with the multispecific recognition capability that P-gp exhibits for transporting drugs and toxins. This finding was subsequently confirmed by measuring the cellular accumulation of fluorescent phospholipids into MDR CEM/VBL300 cells and the fluorescence remaining after quenching in the outer leaflet, which were both decreased compared with parental cells or using P-gp modulators, showing that PC and PE, but not PS, behaved as substrates for P-gp [14]. Similar data concerning the cellular accumulation of fluorescently labelled PC were observed for other MDR cells (KBV1 and MCFadr), showing intramembrane translocation of PC to be much less efficient than that of Rho123 but comparable to that of doxorubicine (two typical P-gp transport substrates) [106]. The question of a similar handling of long-chain, natural lipids by P-gp remained open. However, in the case of an MDR gastric carcinoma cell line, it was observed that a short-chain fluorescent PS is also subjected to outward translocation mediated by P-gp, and this was extended to endogenous PS by using an annexin V binding assay [107]. Furthermore, it was observed that lectin binding to globotriaosylceramide (Gb_3) induced P-gp-mediated PS externalization accompanied by activation of Rho123 efflux out of Raji cells [108], this being consistent with the presence of P-gp in glycosphingolipid-enriched microdomains, and points to Gb_3 as a lipid raft component possibly related to P-gp activity (see below).

P-gp also appears to be involved in the intramembrane translocation of the main lipids constituting the rafts, SM and cholesterol. Indeed, a short-chain fluorescent analog of SM was shown to accumulate in the outer leaflet of cells (CHO, HepG2 and HeLa) having their intracellular vesicular traffic blocked, and this accumulation was inhibited by P-gp modulators [109]. An *in vitro* demonstration of the lipid translocase function exhibited by P-gp was provided more recently by quenching measurements of the leaflet distribution of various NBD-labelled phospholipids, performed on proteoliposomes reconstituted with purified P-gp (in inside-out configuration with respect to the cell) and using P-gp modulators for control: ATP hydrolysis induced PC, PE, PS and SM translocation to the exoplasmic leaflet, independent of acyl chain length and saturation, the clearly highest rate being observed for SM [110]. The same technique was subsequently used for demonstrating P-gp translocase activity for GlcCer and GalCer, and with a lower efficiency for LacCer [111]. It should be mentioned that previous and somewhat similar experiments – but performed on secretory vesicles prepared from *mdr1a*-transfected yeasts (sec6-4 mutant) – did not show any active translocation of NBD-PC, in contrast with *mdr2* (the murine ortholog of the human *MDR3* gene, see below) [112]. Explanations for this discrepancy among the different experimental models include an insufficient level of protein expression in the yeast [113], technical difficulties (dithionite quenching, labelled-lipid donor liposomes and so on) [110], and a difference in lipid composition of the membrane surrounding P-gp (particularly concerning sterols).

Among a series of steroids tested for their uptake into MDR colon carcinoma cells, cholesterol had reduced accumulation compared with parental cells, thus indicating P-gp-mediated active efflux like the other steroids (except progesterone, the most hydrophobic in the series), which all had in addition the capacity to more or less inhibit vinblastine transport by P-gp [12]. This was consistent with the effects observed for the same range of exogenous cholesterol concentrations, increasing P-gp AT-

Pase activity and inhibiting P-gp-mediated daunorubicin transport [77]. Agosterol A, a sterol of marine origin, also displayed the typical properties characterizing a P-gp transport substrate, which are inhibition of P-gp-mediated drug transport and of P-gp photolabelling [114], as well as reduced accumulation in MDR cells, specific photolabelling of P-gp and active transport into P-gp-containing vesicles [115]. Finally, use of native membrane vesicles prepared from MDR cells (DC-3F/ADX) and containing high amounts of P-gp in an inside-out configuration, revealed ATP-dependent vesicle association of exogenous radiolabelled cholesterol as well as an ATP-dependent decrease in endogenous cholesterol accessibility to the soluble enzyme cholesterol oxidase, leading to the conclusion that P-gp works as a cholesterol translocase from the cytosolic to the exoplasmic membrane leaflet [79].

P-gp alters global membrane and lipid pool properties

It has been acknowledged for a long time that not only does P-gp overexpression in tumor cells induce drug resistance due to increased efflux of these drugs out of cells, but this overexpression is also associated with various cellular alterations defining as a whole the 'MDR phenotype' [1]. In particular, this concerns some aspects of cell membrane composition and behaviour. However, the data reported are rather dependent on the cell type considered. For example, in P388 cells, increased amounts of SM and cardiolipin with decreased PE and PS were reported in one study comparing resistant and sensitive cells [116], while no significant quantitative differences in lipid composition were reported in another study [117]. A higher degree of acyl group unsaturation, accompanied by higher membrane fluidity, has been reported for resistant versus sensitive glioma cells but not for transformed liver cells [118]; for the glioma cells, this difference was attributed to increased fatty acid cellular uptake [119]. In contrast, two MDR cell lines (derived from MCF-7 and LoVo) showed a greater amount of esterified cholesterol and triglycerides with increased saturated fatty acids when compared with their sensitive counterparts [120], which could be linked to a greater production of fatty acids in these cells [121]. Also, using proton nuclear magnetic resonance (NMR) analysis of cell lipids, two MDR K562-derived cell lines showed a lower unsaturation level of fatty acids and a higher PC content than in sensitive cells [122]. However, in resistant P388 cells, treatment by MDR-reversing agents induced an increase in PC synthesis, and MDR-linked alteration of membrane lipid composition (and possibly organization) was also revealed by relative resistance to cell permeabilization by digitonin which could be reversed by verapamil [123]. This could be interpreted a posteriori by P-gp-induced alteration of the cholesterol amount and/or distribution within the cell membrane. This is clearly consistent with the recent observation of a lower rate of cholesterol depletion by MβCD in MDR versus sensitive K562 cells [43]. Conversely, cholesterol efflux to MβCD was clearly increased in stably transfected LLC-MDR1 cells but not in conditionally transfected HeLa-MDR/Tet cells [124]. Such data showing (cell type-dependent) alterations of cholesterol exchange with the external medium provides an indication of a possible functional connection between cholesterol intramembrane distribution, cholesterol exchange outside the membrane and intracellular cholesterol trafficking (see below). Indeed, this could also be put in line with the observation that, in human ovarian cancer cells A2780, the total cholesterol content in membranes is somehow inferior in resistant cells than in sensitive (mainly due to less esterified cholesterol), while under cholesterol starvation culture conditions there is only a moderate decrease of cholesterol in resistant cells compared with a large decrease in the sensitive cells which led to a cholesterol level even lower than that in the resistant cells: these data showed clear modification of cholesterol homeostasis in these MDR cells [80]. Finally, lipid NMR analysis of MDR K562 cells has shown no modification of cholesterol content and fatty acid composition with respect to the sensitive cells (the modifications of phospholipid composition being independent of the resistance level): this indicated that MDR-dependent alterations of lipid NMR signals observed in whole cells should be attributed to differences in lipid organization or localization within the cell [125]. The functional connection with raft microdomains remains to be analyzed with regard to possible involvement of P-gp.

P-gp has an influence on endocytosis and membrane traffic/lipid exchanges

A marked increase in adsorptive endocytosis rate, of 2 to 4 times, respectively was observed on two MDR cell lines, of epithelial (EHR) and lymphoid (P388) origins, compared with sensitive counterparts. This finding was attributed to increased internal membrane recycling since the resistant and sensitive cells have the same surface area, and is consistent with increased endosomal volume as evaluated by ultrastructural analysis [126, 127]. Subsequently, it was reported that MDR CEM cells displayed a higher rate of secretion of lysosomal enzymes than their sensitive counterparts, and that this exocytosis stimulation was reversed by verapamil simultaneously with drug resistance [128].

Another example of how the MDR phenotype affects lipid cell metabolism comes from the exchange of lipids, particularly cholesterol, with the external medium. LDL cell uptake and degradation were shown to be clearly lower in MDR cells than in sensitive cells, but this differ-

ence was not removed by verapamil addition to the cell culture medium [80]. However, increased LDL receptor expression was reported on some MDR cells, but this was not changed by P-gp modulators, and it was also reported for some sensitive cells; of particular note, LDL receptor expression is less sensitive to sterol-induced downregulation in MDR cells than in sensitive counterparts [129]. These last observations may be consistent with the fact that in tumor cells, cholesterol level is (generally) regulated via the influx from LDL (i.e. type A cells), while in MDR cells, cholesterol level is (generally) regulated via the efflux to HDL (i.e. type B cells) [105]. Although the exact mechanism involving P-gp remains to be clarified, such a metabolic 'adaptation' of MDR cells is accompanied by an increase in caveolae at the cell surface, which would imply an increase in internal membrane turnover. This could have as a consequence the possibility, as recently observed, of intercellular transfer of P-gp within microparticle-like lipid-protein assembly originating from membrane microdomains [130]. Another possible consequence of increased cell membrane turnover in P-gp-containing cells may be the high efficiency of uptake of cholesterol observed when it was presented to *MDR1*-transfected intestinal cells in mixed micelles [131]. Such a view would explain how P-gp can indirectly mediate flux of a hydrophobic molecule in the inward direction to cell interior, which seems paradoxical with regard to its 'normal' outward floppase function; however, it cannot be excluded that P-gp presence could induce the increased expression of a cell surface protein catalyzing the transfer of cholesterol from the external milieu. In any event, such alterations in lipid traffic in the cell may reveal the influence of P-gp functioning on the internal membrane turnover responsible for the complex process of 'cell lipid sorting', in particular concerning cholesterol [132, 133].

P-gp participates in cholesterol cellular traffic and metabolism

Intracellular cholesterol traffic is a complex process aimed at maintaining cholesterol homeostasis in the cell, and it involves specific internal storage linked to cholesterol esterification; thus exchanges between plasma membrane and internal membranes are of importance, and they are under the influence of microdomains such as caveolae [134]. A connection with MDR has been proposed on the basis of the observation of P-gp-dependent upregulation of the caveolae-mediated cellular cholesterol efflux pathway (see above) [105]. P-gp activity has also been suggested to be linked with favored cholesterol biosynthesis [135] and esterification [136] on the basis of a decrease in these metabolisms when cells (CHO) are treated by various amphiphilic molecules that are mainly P-gp modulators. These two processes could both result from P-gp playing a positive role in cholesterol transfer from the plasma membrane to the ER. The cholesterol biosynthesis which would be favored as a consequence would in turn induce the arrival of newly synthesized cholesterol at the cell surface, and this is in line with the observation of decreased apolipoprotein B secretion nduced by P-gp modulators on Caco-2 cells [137], although the exchanges of cholesterol between plasma membrane and ER are not subject to the same regulation in the two directions [138]. However, despite observation of a facilitating role for P-gp in cholesterol traffic from plasma membrane to ER in both *MDR1*-transfected and drug-selected cells, this could not be correlated with the drug transport inhibiting efficiencies of various amphiphilic P-gp modulators [139]. Also, when comparing KB MDR cells with sensitive cells, there was no correlation observed between modulation of P-gp-mediated drug transport and inhibition of cholesterol esterification [140]. Accordingly, the P-gp inhibitor GF120918 inhibited cholesterol esterification neither in HepG2 nor in MDR MCF-7/ADR cells [141]. However, the *in vivo* consequences of the possible role of P-gp on cholesterol traffic observed in cultured cells are still unclear. In P-gp *mdr1a*-/*mdr1b*-disrupted mice reduced accumulation and increased esterification of cholesterol administered orally, but not intravenously, has been reported in liver but not in other tissues, when compared with wild-type mice [142].

Finally, concerning cholesterol metabolism, findings show increased sensitivity of P-gp-expressing neuroblastoma cells to the cytotoxic action of lovastatine, an inhibitor of the key enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [143]. Further work reported that such higher sensitivity to lovastatin, compared with parental sensitive cell lines, of MDR myeloid leukemia cell lines was accompanied by alteration of cholesterol biosynthesis consisting in increased expression of HMG-CoA reductase and loss of its lovastatin-induced upregulation [144].

P-gp may be involved in ceramide metabolism

Ceramides are lipids constituting membrane microdomains but are also present in internal membranes, where they are biosynthesized. An increased level of GlcCer was first described in three different MDR cell lines (MCF-7-AdrR, KB-V1 and OVCAR-3) compared with their sensitive counterparts [145], and this was restored by various MDR reversing agents [146]. The same data were subsequently confirmed, with additional microfluorometric analysis showing that GlcCer preferentially accumulates in cytoplasmic droplets [147]. A recent study comparing various MDR cell lines (derived from KB and MCF-7) showed good correlation between the expression levels of P-gp and of GlcCer synthase [148]. However, in ovarian cancer cells, it was shown that the elevated level of Glc-

Cer in the MDR versus the sensitive line, accompanied by a reduced level of LacCer, was due to altered intracellular localization of the biosynthesis enzyme LacCer synthase [149]. In contrast, in *MDR1*-transfected HepG2 cells, LacCer is prominently elevated compared with control cells, along with more moderate increases in GlcCer, ganglioside GM3 and SM levels. These alterations were unaffected by cyclosporin A but accompanied by upregulation of LacCer synthase [150]. Nevertheless, another *MDR1*-transfected cell line (MDCK) showed dramatic accumulation of the glycoceramide Gb_3 which was independent to Gb_3 synthase but correlated with P-gp activity [151]. LacCer and Gb_3 synthesis dependent on P-gp functional activity was subsequently observed in various P-gp-expressing cell lines, and it was proposed that P-gp ensures a translocase role for GlcCer at the level of the Golgi membranes [63]. Furthermore, observation of the requirement in Gb_3 expression for a cell to exhibit lectin-induced stimulation of P-gp activity for both PS externalization and Rho123 efflux indicates a functional relationship between P-gp and Gb_3 [108], even though this remains to be clarified. This might be consistent with a general positive role for P-gp in glycolipid biosynthesis and intracellular traffic.

Finally, MDR cells (drug-selected or *MDR1*-transfected) were observed to be sensitive to the pro-apoptosis action of the GlcCer synthase inhibitor PDMP, whereas control cells were not, and this seems to be related to an effect on ceramide metabolism rather than direct action on P-gp-mediated cytotoxic drug transport [152]. Indeed, the pro-apoptotic effect on sensitive cells of GlcCer synthesis inhibition depends on the cell type considered, varying from moderate on MCF-7 [90], marginal on HepG2 [86], to null in melanoma cells [153], and even to a cell-protective effect on leukemic cells [154], whereas it is marked on MDR cells [88].

Other physiological roles for P-gp related to membrane microdomain functions

As exposed in an elegant review, P-gp appears to exhibit an increasing number of physiological roles beside its cell detoxification function against xenobiotics, including lipid traffic and metabolism, immune response, cell proliferation/differentiation and death [155]. Here we will focus on cell processes related to P-gp for which membrane microdomains are reported to be more or less directly involved.

Signal transduction involving the SM/ceramide pathway for apoptosis induction

The SM/ceramide signal transduction pathway has been reported as a metabolic cascade leading to cell death under various cell stress conditions [156]. In the case of cytotoxicity induced by tumor necrosis factor α (TNFα), it has been demonstrated that induced apoptosis depends on the SM pool in the cytosolic leaflet of plasma membrane, and that this pool could be increased by a P-gp modulator such as PSC833 [157]. A further indication of P-gp participation in an apoptotic pathway was provided by the pro-apoptotic effect of the inhibiting P-gp-specific antibody UIC2, which was correlated with the protective effect of P-gp on cells treated with exogenous SM [158]. This was consistent with the functioning of P-gp as an SM outward translocase, as reported above [109, 110]. Recent findings shown resistance of MDR cells against ceramide-induced apoptosis, as compared with sensitive counterparts, and this was reversed by P-gp modulators and accompanied by a concomitent decrease of Rho123 accumulation in the Golgi, suggesting a role for P-gp in GlcCer synthesis [159]. However, P-gp also appears to play a role, still unclear, in different forms of apoptosis cascade activation [160–162], although the connections between membrane microdomains and activation of apoptotic pathways, too, are still rather vague [163–165].

Secretion of biologically active peptides

P-gp expression appeared to be linked with certain physiologic responses of immunologically competent cells, such as dendritic cell migration [166], cytokine secretion [11, 167] and inflammatory response [168]. However, the role and even the participation of membrane microdomains in these phenomena of cytokine secretion and signalling are still poorly described, even if they are often suspected [169], making difficult to rely a defined involvement of P-gp with rafts in this context. However, a general role for rafts in constitutive and regulated secretion processes has been proposed [170].

Recently, it was demonstrated *in vitro* that β-amyloid peptides, involved in Alzheimer disease when secreted in brain, can interact with and be transported by P-gp [171]. This finding has been confirmed *in vivo* in P-gp-null mice, which eliminated microinjected β-amyloid peptides from the brain more slowly than WT mice, and in amyloid precursor protein-expressing transgenic mice, which presented elevated levels of brain β-amyloid peptides when P-gp was altered [172]. Interestingly, the cleavage of the amyloid precursor protein which leads to β-amyloid peptide generation was recently shown to depend on its presence in lipid rafts [173]. This finding may be consistent with data concerning another case of protein misfolding involved in pathological events, and leading to Creutzfeldt-Jakob disease. Indeed, the prion protein is found to reside in caveolae-like domains, where its conversion to the scrapie isoform is determined after proteolytic cleavage [174]. Of course, it remains to be determined whether membrane microdomains play a general role in the biological processing of the amphiphilic peptides produced by pathogenic proteolysis [175].

Interference with viral infections

In two companion articles, it was simultaneously reported that P-gp could have a protective effect against cell infection by some viruses. Actually, MDR cells display significant resistance to enveloped viruses, such as human immunodeficiency virus (HIV), influenza virus and herpes virus, which penetrate cells by fusion with the plasma membrane [176]. In the case of T-cell infection by HIV-1, P-gp overexpression decreased virus production by altering the fusion of viral and plasma membranes and further steps of the intracellular virus life cycle, without modifying the virus receptors at the cell surface [177]. Actually, these observations might be in line with the facts that the early gene product of HIV, Nef, activates infected T cells via an association with the rafts [178], and that influenza virus is described to interact with its target cells via the membrane rafts when budding out of infected cells [179]. More generally, various enveloped or non-enveloped viruses are now described to display a cellular cycle involving lipid rafts at either of the key steps, entry, assembly and budding [180].

Synthesis and perspectives

This rapid literature overview shows that the multidrug transporter P-gp displays mutual relationships with the plasma membrane lipid microdomains, rafts or whatever semantic definitions given to them, in a twofold and entangled manner, that concerns both how P-gp is influenced by its close lipid environment and how it acts on these microdomains, possibly playing a subsequent role on various cellular processes mediated by these microdomains. First, it is established that P-gp exists in rafts and in non-raft membrane domains as well, yet in proportions depending on the cell in question, the experimental conditions and the method used to assay it (probably revealing different raft-like microdomains). Second, cholesterol appears to have a positive influence on P-gp function, but it is still unclear whether this is a direct effect of free cholesterol in the membrane or an indirect effect mediated by cholesterol-enriched microdomains. Third, when present in such rafts, P-gp displays a somewhat favored activity, and is also in good position to interact with certain protein partners (caveolin, actin and so on) aimed at regulating its activity. Fourth, P-gp is a lipid translocase that handles the lipids constituting the rafts with particular efficiency, but, nevertheless, it also appears to influence all membrane trafficking within the cell.

The exact location of P-gp in the membrane microdomains is possibly a peripheral zone suspected to be enriched in cholesterol, around a highly detergent-resistant microdomain core [19, 32]. Such a location could then reconcile the various reports about the differential solubility with caveolin despite their apparent ability to interact together. A similar proposal has very recently been presented pointing rather to a higher content of sphingolipids in the raft core [181]. But P-gp can also be present in distinct microdomains, of slightly different lipid composition, which could meet and interact (and even mix?) one with the other, under certain conditions [31]. However, a view that could emerge from these data is that P-gp should not be considered to be in- or outside lipid rafts, but that it can rapidly shuttle into and back out, in agreement with the notion applied to rafts of highly dynamic microdomains within the membrane. Moreover, this would be favorable for targeting P-gp for various functional regulations, necessarily of transient nature, mediated as well by the lipid composition that it encounters, as a chemical-physical modulation, and by the possible protein partners interacting specifically with it. An example of such specific regulation is P-gp phosphorylation induced by certain protein kinases, even if they are not yet isolated and have a complex and controversial functional role on P-gp [182]; moreover, their relationship with rafts has been proposed [91], although they deserve to be investigated. However, such post-translational regulation of its activity suggests that P-gp is of importance in cell physiology.

As a lipid translocase (like aminophospholipid translocase and scramblase [183]), whatever its exact endogenous substrate(s), P-gp necessarily displays complex and mutual functional relationships with the surrounding lipid phase. In addition, this raises the question of functional coupling between the membrane asymmetry it creates and the lateral heterogeneities where it evolves. Specifically concerning P-gp, its structure may be homologous to that of its related bacterial ABC lipid transporter MsbA [184], and its transmembrane domain would present, at least for some intermediates during its enzymatic cycling, an unusual V-shape, or 'open form', which would alternate with a 'closed form' [185, 186]. Such a large amplitude transconformation of the part of transmembrane domain of the protein which is embedded in the cytosolic leaflet, concomitenly with a rather fixed hedge region of the protein in the exoplasmic leaflet, would be particularly suited to a membrane structure presenting a transversal fluidity gradient as in the rafts [28]. In any event, P-gp translocase functioning participates by itself to the transversal heterogeneity of the membrane, since it affects cholesterol and SM (and possibly ceramides) asymmetry in the membrane. Due to its working as an active transport coupled with ATP hydrolysis, it is assumed to create and maintain a non-equilibrium steady state in the membrane bilayer, while sensing variations in local conditions. As so, it might participate to the complex and inherently dynamic membrane processes such as trafficking and lipid sorting in the cell.

However, P-gp is also (and above all!) recognized as a multidrug transporter responsible for drug and poten-

tially toxic amphiphilic molecule efflux out of the cell. The question of interest is then whether (and how) its drug transport function is influenced by its presence or absence in the rafts. First, this influence obviously depends on the preferential presence of the relevant substrate in either of these two regions, which may alter its partition in the plasma membrane. Second, the rate of transmembrane flux that it mediates may depend on both lipid structure, i.e. raft or non-raft, and on lipid composition, for example presence of cholesterol, as well. These respective characteristics remain to be clarified. In particular, it is not yet known whether drugs and lipids, all being P-gp substrates, display mutual competitive relationships between them or non-competitive interactions (and this probably depends on the chemical structure of the drug). At any rate, it appears that P-gp is able to regulate its own lipid environment and hence its drug transport function – an unusual property for a membrane protein.

Another question that has not been unambigously answered is whether P-gp is able to actively translocate virtually all the lipids constituting a biological membrane, according to its well-known multispecific recognition ability. That is, can it recognize various headgroups and different acyl chains (more or less long and saturated)? Or does it only handle a 'primary' endogenous substrate, which would likely be cholesterol on the basis of its main participation in the basal ATPase activity of P-gp, and which would eventually passively drive other endogenous lipids from one leaflet to the other as a consequence of the created membrane asymmetry?

In any event, it seems that lipids preferentially outwardly translocated as a consequence of P-gp functioning are the components of the rafts. This leads to the notion that P-gp may play a role promoting the formation or maintenance of these rafts, building them by feeding them via increased local concentration of cholesterol and/or SM. In the case of a preferential location of P-gp at the periphery of the microdomains, P-gp functioning could be actively or indirectly involved in their regulation, for example during their coalescence, described to be involved in signal transduction, since this phenomenon would change the ratio periphery/area of these domains with new constraints on local lipid composition of the exoplasmic leaflet. Alternatively, the function of lipid translocase may be modulated by the location of P-gp within or outside the rafts. However, the fact that P-gp functioning favors the existence of rafts in cell membranes leads by itself to the important consequence that P-gp may have, even indirectly, a determining role in all the cellular processes mediated by rafts. Obviously, this deserves further attention to be tested and analyzed. As another indirect, but potentially biologically relevant, effect of P-gp working as a cholesterol floppase, it could be envisioned that it implies a secondary intramembrane displacement of ceramides, which have been observed (in model membranes) to be 'competitive' with cholesterol for their presence in rafts [187].

In this vein, all the hallmarks of the MDR phenotype concerning membranes and lipids in the cell must be questioned about the actual role of P-gp, either direct as a consequence of lipid translocation, or semidirect as a consequence of raft formation, or indirect as a consequence of alteration of local membrane structure and/or dynamics. However, the experimental difficulties in tackling such questions are numerous: a generally marked cell dependence (presence of caveolin, expression level of P-gp, healthy cells vs. tumor cells and so on), an absence of specific inhibiting agents, always possible gene regulation induced by cell stresses concerning, for example, lipid biosynthesis or involvement of other lipid translocase activities.

From a broader point of view, the cellular role of P-gp is linked to the general question of the relationships between membrane asymmetry, lateral heterogeneity and cellular lipid traffic. In particular, manipulations such as depletion and repletion of cholesterol or SM, whose cell metabolisms are known to be closely coupled, in the exoplasmic leaflet from the external medium are reported to induce endocytosis, as a cell response to plasma membrane perturbation, even if the molecular mechanisms involved remain still elusive [20, 170, 188–190]. Along these lines, a possibility would be to consider that the translocase functioning of P-gp creates a transversal membrane asymmetry which could be responsible for increased membrane traffic; of course, this can also be a more direct consequence of the presence of microdomains mediating such actions on their own [133, 134, 170, 191, 192]. Considering the whole level of the cell, P-gp could thus be involved as well in local lipid translocation (cholesterol and SM in the plasma membrane, GlcCer in the Golgi) than in the different steps of membrane and cholesterol trafficking: endocytosis (non-specific or from LDL), transfer to the ER (favoring cholesterol esterification), transfer from the Golgi to the plasma membrane (with precaveolae) and exocytosis (to HDL). As illustrative schemes, see Figure 5 for cell cholesterol trafficking and Figure 3 in the recent review of van Meer and Sprong [20] for ceramide biosynthesis.

As a final word, it should be mentioned that such a theme is likely to be applicable to the other ABC multidrug transporters, MRPs and MXR/BCRP, for which studies about functional relationships with their lipid environment are just starting. Briefly, for MRP1 (ABC C1, also responsible for the MDR phenotype), findings in erythrocytes show translocase activity for NBD-labelled PS and PC [193, 194]. In *MRP1*-transfected epithelial kidney cells, a translocase activity for NBD-labelled GlcCer and SM, requiring glutathione, has also been described at the basolateral membrane [195]. Moreover, MRP1 was

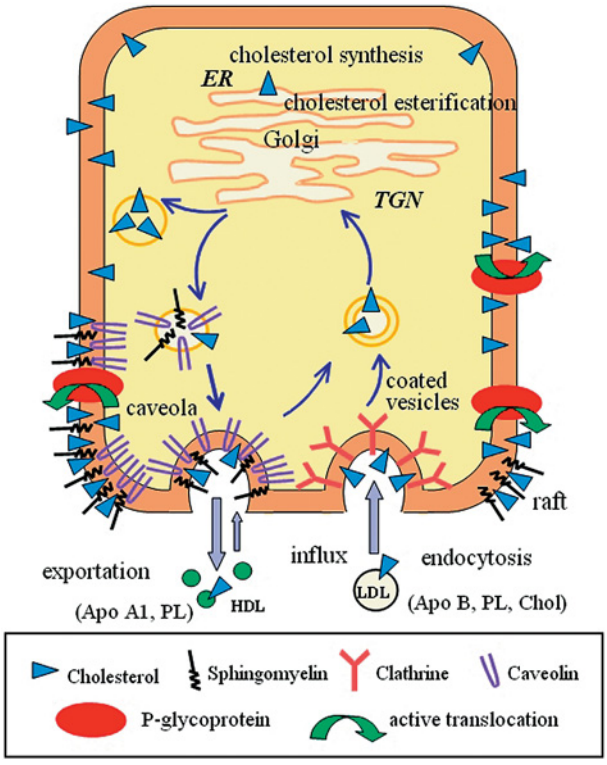


Figure 5. Functional scheme of P-gp involvement in cellular cholesterol trafficking. In cells, according to an oversimplified view, the cholesterol amount is regulated between endogenous cholesterol biosynthesis and esterification in the ER, an exogenous cholesterol import from LDL by endocytosis and a cholesterol export to HDL [133, 134]. The active cholesterol flux mediated by P-gp from the cytosolic to the exoplasmic leaflet of the plasma membrane supports a role for P-gp in cholesterol enrichment of rafts and caveolae, leading to increased integration of caveolin-1 in plasma membrane, and possibly to upregulation of other steps in intracellular cholesterol trafficking. (ER, endoplasmic reticulum; TGN, trans-Golgi network.)

preferentially found in the Lubrol-insoluble membrane domains compared with the Triton X100-insoluble domains, independent of caveolin [50]. Finally, in MRP1-overexpressing cells, GlcCer biosynthesis was upregulated concomitently with the drug resistance level [196]. As regards MXR (ABC G2), it is worth mentioning that it has been reported to have activity modulated by sterols and steroids and to transport estradiol [197]. More generally, there are also ABC transporters devoted to the transport of different lipids with rather higher selectivity: MDR3 (ABC B4) is a PC translocase involved in bile secretion [112, 198], ABC D1–4 are involved in very long chain fatty acid transport and metabolism in peroxisomes [199], CFTR (ABC C7, whose mutation is responsible for cystic fibrosis) regulates cellular uptake of sphingosine 1-phosphate [200], ABC A1 is responsible both for PS externalization [201] and for the transfer from plasma membrane to apolipoprotein-A1 of phospholipids and cholesterol [202], ABC G1 is involved in cholesterol homeostasis in macrophages [203], and the heterodimer ABC G5/G8 is responsible for intestine and hepatobiliary secretion of cholesterol and dietary sterols [204, 205]. More details are provided in recent reviews [113, 206, 207].

In conclusion, the present state of the art provides some information 'ready to use' but also reveals many aspects requiring deeper analysis and further clarification from the different perspectives of membrane biophysics, lipid biochemistry and cell physiology of signal transduction. This will undoubtedly be an open and lively field of active research in the near future, aimed at developing both physiological knowledge and pharmacological applications.

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