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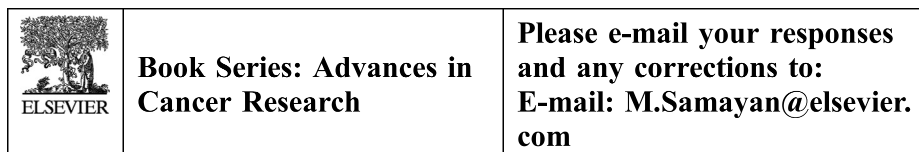

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Lipid Regulation of the ABCB1 and ABCG2 Multidrug Transporters

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Abstract

This chapter deals with the interactions of two medically important ABC multidrug transporters (MDR-ABC), ABCB1 and ABCG2, with lipid molecules. Both ABCB1 and ABCG2 are capable of transporting a wide range of hydrophobic drugs and xenobiotics and are involved in cancer chemotherapy resistance. Therefore, the exploration of their mechanism of action has major therapeutic consequences. As discussed here in detail, both ABCB1 and ABCG2 are significantly affected by various lipid compounds especially those residing in their close proximity in the plasma membrane. ABCB1 is capable of transporting lipids and lipid derivatives, and thus may alter the general membrane composition by "flopping" membrane lipid constituents, while there is no such information regarding ABCG2. Still, both ABCB1 and ABCG2 show complex interactions with a variety of lipid molecules, and the transporters are significantly modulated by cholesterol and cholesterol derivatives at the posttranslational level. In this chapter, we explore the molecular details of the direct transporter–lipid interactions, the potential role of lipid-sensor domains within the proteins, as well as the application of experimental site-directed mutagenesis, detailed structural studies, and *in silico* modeling for examining these interactions. We also discuss the regulation of ABCB1 and ABCG2 expression at the transcriptional level, occurring through nuclear receptors involved in lipid sensing. The better understanding of lipid interactions with these medically important MDR-ABC transporters may significantly improve further drug development and clinical treatment options.

st0015 **ABBREVIATIONS**

dt0005 **CHAPS** 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate Au3
dt0010 **CMC** critical micelle concentration Au4
dt0015 **DMPE** 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine
dt0020 **DPPE** 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
dt0025 **ESR** electron spin resonance
dt0030 **β-MCD** β-methyl cyclodextrane
dt0035 **MDR-ABC** multidrug resistance ATP-binding Cassette
dt0040 **NMR** nuclear magnetic resonance
dt0045 **PC** phosphatidylcholine
dt0050 **PE** phosphatidylethanolamine
dt0055 **SDS** sodium dodecyl sulfate

s0005
1. INTRODUCTION—THE COMPLEX INTERACTIONS OF LIPIDS AND ABC MULTIDRUG TRANSPORTERS

p0060 The subjects of this chapter are the interactions of two clinically important ABC multidrug (MDR-ABC) transporters, ABCB1 (P-gp/MDR1) and ABCG2 (ABCP/BCRP/MXR), with their membrane lipid

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environment and other lipid molecules. Since several chapters in this book present detailed descriptions of MDR-ABC transporters, here we only shortly summarize their main features.

p0065 Both ABCB1 and ABCG2 are glycosylated integral plasma membrane proteins which are involved in the cellular extrusion of a large variety of xenobiotics and endobiotics. These ATP-dependent transporters contain large transmembrane domains (TMDs) composed of alpha helices and similarly to all other ABC transporters, cytoplasmic nucleotide-binding domains (NBDs). In a general mechanism of action, the binding and hydrolysis of ATP in the NBDs provide the energy for drug or xenobiotic extrusion through the transmembrane regions of the protein. ABCB1 has been the first recognized plasma membrane multidrug transporter, which contains 2 NBDs and 2 TMDs with altogether 12 transmembrane helices within 1 polypeptide chain. The ABCG2 protein was discovered only about 15 years ago and is a "half ABC transporter" containing only one TMD consisting of six transmembrane helices and one NBD. The functional form of ABCG2 has been shown to be a homodimer or homo-oligomer (Bhatia, Schafer, & Hrycyna, 2005; Ni, Mark, Cai, & Mao, 2010; Ozvegy et al., 2001; Xu, Liu, Yang, Bates, & Zhang, 2004).

p0070 Both of these transporters have a "promiscuous" capacity of recognizing a large number of transported substrates. The ABCB1 protein transports mostly hydrophobic or positively charged amphipathic molecules, while the substrate specificity of the ABCG2 transporter is even wider and includes entirely hydrophobic toxic compounds, amphiphilic positively or negatively charged, as well as practically water-soluble molecules. Besides their capability of transporting various therapeutic drugs, these MDR-ABC transporters also play an important physiological role in cellular and systemic detoxification and are key members of the xenobiotic or the so-called chemoimmunity defense system of the body (Sarkadi, Homolya, Szakacs, & Varadi, 2006; Sarkadi, Muller, & Hollo, 1996). Besides its general protective role, ABCB1 is currently not known to transport any specific physiological substrates. In contrast, ABCG2 has recently been shown to be an efficient uric acid transporter and thus plays an important role in urate metabolism. Polymorphic ABCG2 variants are strongly implicated in the development of gout (Woodward et al., 2009).

p0075 Both ABCB1 and ABCG2 are preferentially expressed in various tissue barriers and in the apical/luminal membranes of polarized cells. In cancer cells, the expression of these MDR-ABC transporters results in cross-resistance (or multidrug resistance, MDR) against a wide range of

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chemotherapeutic drugs, including practically all available anticancer agents that have to cross the cell membrane to reach their intracellular targets. Moreover, normal and cancer stem cells express ABCG2 (and in some cases ABCB1), and this expression pattern may be an important factor in the inherent chemotherapy resistance of cancer stem cells.

p0080 As mentioned above, both ABCB1 and ABCG2 reside in the plasma membrane and in polarized cells are recruited into the apical (luminal) membranes. The mammalian plasma membrane has a complex structure of several lipid species in which selected lipids may preferentially surround membrane proteins. Membrane lipids are known to greatly affect the structure and function of a number of membrane proteins, especially those interacting with hydrophobic or amphipathic substrates. Even in the cases of those membrane proteins, e.g., ion channels or hormone receptors, when the ligands are hydrophilic species, protein conformational changes are required to transmit the messages or ions through the membrane environment, and the energetics and speed of this information or material transfer may be greatly affected by lipid interactions (Laganowsky et al., 2014; Phillips, Ursell, Wiggins, & Sens, 2009). Basic categories of these physical and/or chemical interactions are summarized in (Fig. 1). The membrane effects on integral proteins are amplified by the formation of various lipid microdomains, depending on lipid compositions, temperature, and membrane-associated (e.g., cytoskeletal) proteins.

p0085 The annular lipid regions around transmembrane regions of membrane proteins may directly modulate their activities. The special membrane domains showing different lipid and protein associations, in most cases

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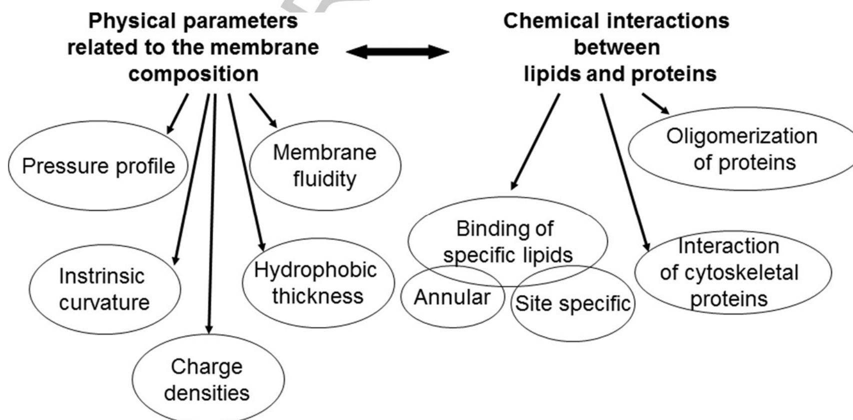

f0005 **Figure 1** Membrane parameters potentially modifying membrane protein function.

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enriched in sphingomyelin (SM) and cholesterol are often called lipid rafts. In biological membranes, in contrast to unsaturated glycerophospholipids, SMs in the outer plasma membrane leaflet in many cases have stretched conformations, and this SM conformation allows more intimate contacts with cholesterol. Also, the larger headgroup of SM may shield the hydrophobic part of cholesterol from water; thus, SM and cholesterol may become closely packed in a partially ordered phase (Ohvo-Rekila, Ramstedt, Leppimaki, & Slotte, 2002; van Meer, Voelker, & Feigenson, 2008). The definition of such lipid rafts is quite variable and sometimes controversial (Mayor & Rao, 2004; Munro, 2003; Simons & Gerl, 2010)—biochemists and cell biologists have somewhat different approaches in this regard. Biochemists regard lipid rafts as the membrane fractions which are insoluble or less soluble in nonionic detergents, e.g., Triton X-100, CHAPS, Brij 96, or Lubrol WX (London & Brown, 2000), although even these different detergents produce variable "raft" fractions. In this regard, Brij 96 and Lubrol WX have milder solubilizing potential than Triton X-100 (Schuck, Honsho, Ekroos, Shevchenko, & Simons, 2003); thus, "Lubrol rafts" may contain different membrane lipids and proteins than "Triton rafts." Cell biologists are more concerned about close proximity selective protein interactions, observed in 20–100 nm wide dynamically organized membrane regions under various functional conditions, including membrane trafficking, receptor-dependent signal transduction, channel formation, or substrate transport (see Fig. 1).

p0090 As agreed by both the biochemistry and cell biology approaches, lipid rafts in the plasma membranes contain most of the glycosylphosphatidylinositol-anchored proteins, gangliosides, and flotillin and may pull together a variety of proteins involved in the signal transduction machinery (Pike, 2003). A specialized type of raft domains is the caveola, characterized by membrane invaginations stabilized by a hairpin-like membrane protein, caveolin. Cytoskeletal elements at the internal membrane surface may stabilize the rafts and the included integral membrane proteins, and such a "membrane scaffold" has been implicated in numerous membrane protein functions.

p0095 Both ABCB1 and ABCG2 have been reported to reside in detergent-insoluble rafts (Ismair et al., 2009; Orlowski, Martin, & Escargueil, 2006; Radeva, Perabo, & Sharom, 2005; Storch, Ehehalt, Haefeli, & Weiss, 2007) and in close interaction with cholesterol and caveolin. In the canalicular membranes of hepatocytes, several ABC transporters, including ABCB4 (MDR3), ABCB11 (BSEP, S-P-gp), ABCC2 (MRP2), and ABCG5/G8, have also been shown to reside in raft domains (Ismair et al., 2009). The properties of transmembrane regions of ABC transporters, such as the length

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of transmembrane helices and/or the interaction of transmembrane helices with cholesterol, may actually favor the localization of these proteins in the raft domains.

p0100 It is important to note that ABCB1 is in a mutual relationship with its membrane environment, that is, not only the membrane lipids alter the function of ABCB1 but this protein may actively modify its lipid environment (for reviews, see Denning & Beckstein, 2013; Orlowski et al., 2006; Peetla, Vijayaraghavalu, & Labhasetwar, 2013; Pohl, Devaux, & Herrmann, 2005; Sharom, 2014). ABCG2 is less characterized in this respect, but lipid interactions certainly affect its function.

p0105 In studying the lipid regulation of ABC membrane transporters, the selection of proper assay systems is a major hurdle. Since MDR-ABC transporters have key interactions with a wide range of hydrophobic toxins and drugs, it is inherently difficult to analyze the lipid effects on the actual substrate transport pathway or on the general conformation and activity of the protein. Clearly, membrane lipid constituents, especially cholesterol, may act both ways. In whole cell assays, studying drug resistance or direct drug/xenobiotic extrusion, lipid modulation may be easily recognized by the altered function of the transporter, but lipid modifications have to be properly controlled not to cause a general damage of the plasma membrane composition. Lipid enrichment or extraction methods thought to be selective may result in a nonspecific permeabilization or a general disruption of the lipid rafts and/or the associated cytoskeletal elements.

p0110 The key functional assays in isolated cell membrane preparations involve the measurement of substrate-stimulated ATPase activity and the direct transport activity of ABCB1 or ABCG2. Although coupling between the ATPase and drug transport activity is well established, both ABCB1 and ABCG2 have significant basal ATPase activities even in the absence of drug substrates. The nature of this basal ATPase activity has not been properly established. Direct determination of the vesicular uptake of radiolabeled or fluorescent substrates into inverted membrane vesicles may overcome this problem. For vesicular transport studies, the lipid-dependent tight sealing of the vesicles is essential and may be significantly altered by lipid modifications. Both transporters have a well measurable function especially when expressed in insect cells, capable of generating large amounts of functional, membrane-inserted proteins. However, mammalian cell membranes have five to eight times higher cholesterol levels than the insect cell membranes, which may significantly alter ABC transporter activity. A relatively mild way of reducing or increasing cell membrane lipids, especially cholesterol, is the

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incubation of the cells with "empty" or lipid-complexed cyclodextrin compounds (Telbisz et al., 2007; Zidovetzki & Levitan, 2007).

p0115 Another suitable assay system for analyzing the ABC transporter and lipid interactions is based on the preparation of isolated and reconstituted proteins. In this case, the modulation of the lipid composition used for reconstitution allows a direct estimation of the lipid effects. However, these methods are inherently difficult to perform and the activity of the purified ABC transporter may be significantly affected by the remaining detergent or lipid content of the reconstituted proteoliposomes (Decottignies, Kolaczkowski, Balzi, & Goffeau, 1994; Mao, Deeley, & Cole, 2000; Telbisz et al., 2007).

s0010

2. EFFECTS OF LIPIDS ON THE FUNCTION OF ABCB1 AND ABCG2

s0015

2.1. Localization of ABCB1 and ABCG2 in specialized membrane domains

p0120 Since the cholesterol–SM-rich "raft" regions are heterogeneous in composition, size, and timescale of existence (see Section 1), the association of ABCB1 and ABCG2 with these regions has been examined by a number of methods.

p0125 ABCB1 has been reported to reside in Triton X-100-resistant rafts (Ismair et al., 2009; Orlowski et al., 2006) or in Brij 96-resistant rafts (Radeva et al., 2005), although ABCB1 was found to be an active drug transporter both in raft and in nonraft regions (Bucher, Besse, Kamau, Wunderli-Allenspach, & Kramer, 2005). Still, recent studies, examining the ratio of active state conformations of ABCB1 by the conformation sensitive UIC2 antibody, indicate that this protein may be more active in cholesterol-rich, caveolin-positive regions and thus may be regulated by factors altering raft assembly (Bacso et al., 2004). Treatment of cells by β-methyl cyclodextrin (β-MCD) for cholesterol depletion or the application of cholesterol-loaded β-MCD for cholesterol enrichment was reported to modify the transport functions of ABCB1 in several experiments, but in other reports, no significant effects were observed (Luker, Pica, Kumar, Covey, & Piwnica-Worms, 2000; Telbisz et al., 2007). This conditional effect may be dependent on the cell-type and transported substrates examined. Moreover, the application of high concentrations of β-MCD may cause significant nonspecific membrane alteration effects and toxicity.

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p0130 ABCG2 was found in Triton X-100-insoluble lipid rafts in close interaction with cholesterol and caveolin (Storch et al., 2007). As discussed below, in the case of ABCG2, cholesterol is an essential modulator of the transport activity; thus, raft localization may be advantageous for its proper functioning.

p0135 Most intracellular membranes contain relatively low levels of cholesterol; thus, ABCB1 and ABCG2 transporter activity may be significantly lower during membrane protein processing and traveling from the ER and Golgi to the plasma membrane. Moreover, the apical membranes of polarized cells contain significantly more cholesterol-rich microdomains (Simons & Gerl, 2010) than the basolateral regions, and this unequal cholesterol distribution may also result in large differences in transporter activity. Since both ABCB1 and ABCG2 are localized in the apical–luminal compartments in polarized tissue regions (e.g., in the liver canalicular membranes, in the intestinal or kidney epithelia), specific local interactions may be important factors in the lipid regulation of transporter activity in these regions.

s0020 2.2. Substrate handling of ABCB1 and ABCG2 and the role of membrane lipids

p0140 Hydrophobic molecules accumulate in the lipid phase of the cellular membranes and achieve much higher concentrations within the membranes than in the cytoplasmic or extracellular water phases. Cholesterol, in addition to stabilizing membrane structures, may further increase lipid insertion of hydrophobic drugs or xenobiotics. In addition, amphiphilic, positively charged drug compounds, e.g., doxorubicin, mitoxantrone, or verapamil, interact with negatively charged lipid headgroups, while the hydrophobic parts of these drugs interact with apolar membrane regions (Siarheyeva, Lopez, & Glaubitz, 2006; Speelmans, Staffhorst, De Wolf, & De Kruijff, 1995). Therefore, membrane lipids might significantly affect the plasma membrane concentrations and availability of ABCB1 or ABCG2 substrates.

p0145 Practically all of the ABCB1 and most of the ABCG2 substrates are lipophilic/amphiphilic compounds, and it is by now well accepted that these transporters may gain access to their hydrophobic substrates directly in the membrane lipid phase (Coleman, Quazi, & Molday, 2013; Sharom, 2014). Several experimental results indicate that ABCB1 binds its drug substrates within the plasma membrane and most probably in the inner leaflet of the plasma membrane (Clay & Sharom, 2013; Higgins & Gottesman, 1992; Sharom, 1997). Drug partitioning into the lipid membrane and drug binding to ABC multidrug transporters show close correlation (Clay &

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Sharom, 2013; Romsicki & Sharom, 1999). FRET studies, applying a fluorescent substrate drug (doxorubicin) of ABCB1 and a lipophilic fluorescent compound (iodonaphtalen-1-azide) that can be directly attached to ABCB1 through photoaffinity labeling, indicated drug binding to the transporter within the lipid phase (Raviv, Pollard, Bruggemann, Pastan, & Gottesman, 1990).

p0150 In addition, several experimental results imply that both ABCB1 and ABCG2 can expel extracellularly added hydrophobic compounds from the cells before these compounds would reach the cytoplasm. In one of these approaches, ABCB1 was shown to reduce the cellular accumulation of fluorescent indicator dyes. These compounds (e.g., Quin-2, Indo-1, or Fluo-4) are usually added to the cells in a nonfluorescent acetoxymethylester (AM) form, and then intracellular esterases produce the fluorescent, free dye compounds. It has been observed that when the ABCB1 protein is expressed in the cell membrane, the fluorescent dye accumulation is strongly reduced because the transporter removes the AM compound from the membrane before the cytoplasmic esterases could produce the fluorescent free dye (Homolya et al., 1993). Moreover, the active extrusion of calcein-AM, a dye used earlier as a viability indicator (the free calcein fluorescence is insensitive to cellular ionic environment or pH), allowed the development of a highly sensitive fluorescent assay for measuring cellular ABCB1 transport activity (Hollo, Homolya, Davis, & Sarkadi, 1994; Homolya, Hollo, Muller, Mechetner, & Sarkadi, 1996; Homolya et al., 1993).

p0155 Another detailed study of ABCG2 activity documented that mitoxantrone, a cytotoxic agent that is highly fluorescent in the lipid phase, accumulates in the cell membrane but this accumulation is strongly reduced by the presence and activity of ABCG2. Mathematical modeling based on detailed cellular microscopy fluorescence measurements further supported a direct extrusion of mitoxantrone by ABCG2 from the membrane phase (Homolya, Orban, Csanady, & Sarkadi, 2011).

p0160 Thus, hydrophobic drugs are most probably extruded by ABCB1 and ABCG2 from the plasma membrane. Two transport models have been proposed to describe this phenomenon, the so-called hydrophobic vacuum cleaner and the flippase models (see Fig. 2 and Higgins & Gottesman, 1992). In both models, the transported substrate is recognized within the membrane; however, while according to the "vacuum cleaner" model the substrate is released to the extracellular water phase, the flippase model (in recent times modified to "floppase," based on its outward direction) suggests that drugs are only "flopped" by the transporter from the inner to the

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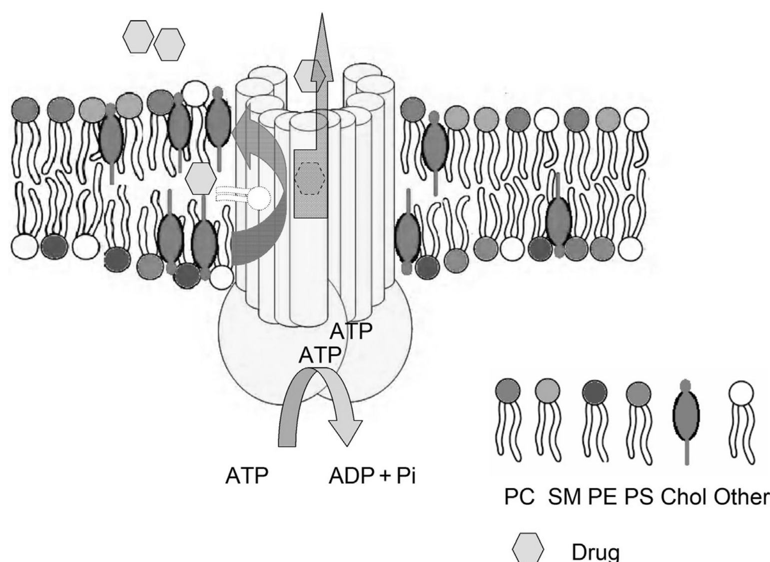

f0010 **Figure 2** *Membrane lipidsand their potential interactions with MDR-ABC transporters.* Lipids may modulate the transport of substrate drugs by several forms of molecular interactions. The figure represents asymmetric lipid distribution in the membrane leaflets and shows possible distortion of the membrane by MDR-ABC transporters and cholesterol enrichment. The red (dark gray in the print version) arrow represents floppase activity (that can be valid for drug and lipid transport as well), while the brown (gray in the print version) arrow represents outward transport ("hydrophobic vacuum cleaner") activity (for details see text).

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outer membrane leaflet and then either diffuse to the extracellular water phase or remain in the outer membrane leaflet. In fact, a significant enrichment in the outer membrane leaflet and a large outward substrate gradient may produce the same extrusion effect than a direct transport to the extracellular fluid. In the future, a more detailed knowledge on the atomic level structure of the transporters may help to distinguish between these molecular mechanisms of action.

s0025 2.3. Modulation of ABCB1 and ABCG2 function by lipids, lipid derivatives, and detergents

p0165 The transport functions of ABCB1 and ABCG2 are significantly affected by membrane lipids especially by cholesterol, as well as lipid derivatives and detergent compounds. ABCB1 in fact is able to transport (or flop) various membrane phospholipids and lipid derivatives (van Helvoort et al., 1996). There are no available data for phospholipid or phospholipid-derivative

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transport by ABCG2, while cholesterol has been shown to display a significant effect on this transporter. Still, due to technical limitations, in most cases, it is difficult to clarify whether a lipid compound is a modulator or a transported substrate of these MDR-ABC proteins. In many cases, even direct lipid or detergent interactions cannot be distinguished from indirect effects, caused by the alteration of the general membrane properties.

p0170 In this chapter, we focus on the interactions of cholesterol and its main derivatives with ABCB1 and ABCG2. However, it is also worth mentioning that several steroid hormones and hormone metabolites have been shown to be transported substrates of these proteins. ABCB1 was indicated to transport progesterone and aldosterone (Kim & Benet, 2004; Ueda et al., 1992), while ABCG2 is an efficient transporter for hormone derivatives, including estradiol sulfate and estradiol glucuronide (Suzuki, Suzuki, Sugimoto, & Sugiyama, 2003).

p0175 Cholesterol depletion of the cell membranes has been reported to decrease both ABCB1 and ABCG2 function (Storch et al., 2007; Troost, Lindenmaier, Haefeli, & Weiss, 2004). For assessing a direct modulation of these transporters by membrane cholesterol, various functional assays should be combined, e.g., measurement of vanadate-sensitive, basal, or drug-stimulated ATPase activity, direct drug transport in isolated membranes, or combined ATPase and transport activity by isolated and reconstituted proteins (see Section 1).

p0180 ABCB1 is most probably not directly involved in cholesterol transport (Tarling, de Aguiar Vallim, & Edwards, 2013), and studies for the cholesterol modulation of the transporter yielded variable results. ABCB1 ATPase activity was measured in most cases in native membranes prepared from ABCB1-overexpressing cells. While the basal ATPase activity of ABCB1 was increased by cholesterol, the substrate-stimulated activity was less modified (Belli, Elsener, Wunderli-Allenspach, & Kramer, 2009; Bucher, Belli, Wunderli-Allenspach, & Kramer, 2007). In addition, in the case of smaller size drug substrates, cholesterol had a much greater influence on the substrate-dependent ABCB1-ATPase activity than in the case of larger drug substrates (Kimura, Kioka, Kato, Matsuo, & Ueda, 2007). Thus, the function of ABCB1 seems to be relatively insensitive to changes in the cholesterol content of the plasma membrane and the effects of cholesterol largely depend on the size of the transported substrate (see in detail in Section 4.1.2).

p0185 Although several close relatives in the ABCG subfamily are involved in steroid transport (ABCG1 transports cholesterol and the ABCG5/G8 heterodimer transports plant sterols), ABCG2 is probably not an active

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cholesterol transporter (Kimura, Kodan, Matsuo, & Ueda, 2007), and no ABCG2 defects have been associated with cholesterol metabolic diseases. Still, cholesterol has a major modulatory effect on ABCG2 activity.

p0190 When ABCG2 is expressed in cholesterol-poor insect cell membranes, the addition of cholesterol in the form of a complex with β-MCD significantly stimulates both drug transport and ATPase activity (Pal et al., 2007; Telbisz et al., 2007). The addition of other sterol compounds, e.g., sitosterol or ergosterol, has similar effect, although the most effective modulator in lower concentrations is cholesterol (Telbisz et al., 2007). Experiments performed using isolated ABCG2 strongly indicate that cholesterol or other membrane sterols are essential cofactors influencing ABCG2 function. In the case of isolated and reconstituted ABCG2, both ATPase and transport activity have been shown to be very low in a membrane lipid environment lacking cholesterol (Telbisz, Ozvegy-Laczka, Hegedus, Varadi, & Sarkadi, 2013). Cholesterol has been shown to increase the basal ATPase activity of ABCG2 in a concentration-dependent manner; moreover, stimulation of the ABCG2-ATPase activity by transporter substrates could only be achieved in the presence of cholesterol (Telbisz et al., 2013).

p0195 The major cholesterol derivatives in the human body are bile salts, and in some tissues, e.g., in the liver and the intestine, where bile salt concentrations are relatively high, these compounds may significantly affect ABC transporter function. Since bile acids and derivatives also have strong detergent activity, this feature may also be involved in their functional effects.

p0200 A close homologue of ABCB1, ABCB11 (BSEP/S-P-gp), is the main bile acid transporter in the liver canalicular membrane, while ABCB1 has not been shown to be involved in bile acid transport. Substrate transport by ABCB1 has been reported to be inhibited by bile acids (Mazzanti et al., 1994), but the detergent effects of bile acids may also explain this phenomenon.

p0205 The function of ABCG2 has been shown to be significantly modulated by bile salts and detergents with chemical structures resembling to bile salts (e.g., CHAPS) (Telbisz, Hegedus, Varadi, Sarkadi, & Ozvegy-Laczka, 2014; Telbisz et al., 2013). In isolated ABCG2-containing membranes or in reconstituted ABCG2 proteoliposomes, bile salts applied in much lower concentrations than the potential detergent effects strongly decreased the basal ABCG2-ATPase activity while not affecting the substrate-stimulated ATPase activity in the presence of cholesterol (Telbisz et al., 2014). Experiments detailed below indicate that both cholesterol and bile acids modulate ABCG2 function independently of interacting with the substrate transport sites.

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p0210 Regarding potential bile salt transport by ABCG2, the experimental results are contradictory. ABCG2 expressed in *Lactococcus lactis* showed higher ATPase activity in the presence of certain bile salts and caused a minor bacterial resistance against bile salt toxicity (Janvilisri, Shahi, Venter, Balakrishnan, & van Veen, 2005). In CHO cells, coexpression of rat Oatp1a1 and human ABCG2 enhanced both the uptake and the efflux of a fluorescent conjugated bile acid, cholyl-glycylamido-fluorescein, cholic acid (CA), glycoCA, tauroCA, and taurolithocholic acid-3-sulfate (Blazquez et al., 2012). In contrast, in our experiments using human ABCG2-expressing isolated insect membranes, we could not detect any ATP-dependent vesicular accumulation of radiolabeled bile acids. Under the same experimental conditions, we could measure substantial vesicular transport by the known professional bile acid transporters, ABCC2 (MRP2) and ABCB11 (BSEP/S-P-gp) (Telbisz et al., 2014).

p0215 Detergents, by disrupting hydrophobic protein interactions with membrane lipids, in most cases, inhibit ABCB1 and ABCG2 function. Strong ionic detergents, such as SDS, may irreversibly destroy transporter function. Still, some nonionic detergents, such as Triton X-100 or C12EO8, below their CMC concentrations, have been indicated to be transported substrates of ABCB1 and possibly bind to the drug-binding site of the protein (Beck et al., 2013; Li-Blatter, Nervi, & Seelig, 2009; Seelig & Gerebtzoff, 2006; Zordan-Nudo, Ling, Liu, & Georges, 1993). Bile acids and related compounds (e.g., CHAPS) modify ABCG2 transporter function below the concentrations causing detergent effects, while some potentially "mild" detergents may have serious adverse effects. Since functional ABCG2 is a homodimer, a detergent-induced dissociation of the ABCG2 dimer has been shown to irreversibly destroy the function of this transporter (Telbisz et al., 2014).

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s0030 2.4. Role of lipids in MDR-ABC protein purification and reconstitution

p0220 Solubilization, isolation, and reconstitution of large membrane proteins are in most cases more related to "art" than to simple technologies, and this is certainly true in the case of MDR-ABC transporters.

p0225 For the *solubilization* of ABCB1, certain mild detergents, e.g., octylglycoside, dodecyl-maltoside (DDM), or CHAPS, were optimal for preserving ABCB1 activity, possibly by retaining a lipid shell around the protein (Ambudkar, 1995; Doige, Yu, & Sharom, 1993; Orlowski et al., 1998; Shapiro & Ling, 1995; Sharom, Yu, & Doige, 1993). Indeed, CHAPS Au5

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molecules were found to be in close association with a partially purified ABCB1 (Sharom, Yu, Chu, & Doige, 1995). DDM alone was unable to maintain functionality, but in the presence of lipid additives (e.g., *Escherichia coli* lipids) solubilization with this detergent also preserved ABCB1 activity.

p0230 In the process of solubilization and isolation of ABCG2, the choice of detergents has been proved to be the most important factor to obtain a functional transporter (Telbisz et al., 2013). Purification trials revealed a high sensitivity of ABCG2 to total delipidation. Therefore, mild solubilization conditions had to be applied in order to preserve a lipid shell around the solubilized ABCG2 protein. The homodimeric ABCG2 protein was even more sensitive to detergents than ABCB1, and the addition of extra lipids at the solubilization step was indispensable for maintaining activity (Telbisz et al., 2014).

p0235 *Reconstitution* studies showed that purified ABCB1 can be functional in several types of liposomes, composed of different phosphatidylcholine (PC) and phosphatidylethanolamine (PE) mixtures, and the addition of cholesterol is not required for full activity (Bucher et al., 2007; Doige et al., 1993; Kimura, Kioka, et al., 2007; Modok, Heyward, & Callaghan, 2004). ABCB1 was active even in pure DMPE or DPPE liposomes, but the application of PC:PE mixtures increased activity and unsaturated lipids also had an activating role (Doige et al., 1993). The analysis of the effects of the length of lipid side chains revealed an optimum bilayer width, and the headgroups of the lipids were also found to be important (Sharom, 2014).

p0240 The presence of cholesterol is not necessary for purified ABCB1 activity in liposomes (Bucher et al., 2007; Kimura, Kioka, et al., 2007), and not only other sterols but also α-tocopherol or even DPPC caused the same moderate effect as cholesterol on the ABCB1 function in PC liposomes (Belli et al., 2009).

p0245 The purified and reconstituted ABCG2 was found to be inactive in PC: PE lipid mixtures and had low activity in an *E. coli* lipid extract, containing no cholesterol. In contrast, the protein had high activity in cholesterol containing natural lipid extracts, e.g., in brain lipids or in *E. coli* lipid extract that was supplemented with cholesterol. The *E. coli* lipid extract was the most suitable for examining the sterol dependence of ABCG2 activity, and these experiments showed that sitosterol or ergosterol can also activate the ABCG2 protein (Telbisz et al., 2007). In Section 4, we provide a detailed analysis of the potential molecular interactions between sterols and ABCG2.

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2.5. MDR-ABC transporters may actively alter the membrane lipid environment

Since ABCB1 and ABCG2 transport hydrophobic compounds, they may significantly modulate the composition of the plasma membrane around their residing areas. Several publications revealed differences in the lipid composition of parental and MDR-ABC-expressing drug-resistant cell line pairs (Hinrichs, Klappe, & Kok, 2005; Peetla et al., 2013). ABCB1 has indeed been shown to be capable of the outward transport of NBD labeled or radiolabeled short PC and PE analogues (Bosch, Dunussi-Joannopoulos, Wu, Furlong, & Croop, 1997; van Helvoort et al., 1996).

This transport in whole cells represents a lipid "floppase" activity of the protein. In liposomes, the purified and reconstituted ABCB1 was also found to have phospholipid and glycolipid transport activity. In liposomes containing purified ABCB1 in an inverted orientation, an ATP-dependent transport activity was found when measuring the movement of fluorescent lipid analogues from outside to the inside of the liposomes. This lipid transport activity was inhibited by ABCB1 drug substrates; thus, drug and lipid transport activities are similar processes (Eckford & Sharom, 2005; Romsicki & Sharom, 2001).

According to data in the literature, ABCB1 can also transport ceramides and sphingolipids (Clay & Sharom, 2013; Coleman et al., 2013; Radeva et al., 2005; Sharom, 2014), indicating a wide substrate specificity of ABCB1 also for lipid molecules. This lipid floppase activity can be involved in altering the cell membranes for drug distribution, thus modifying the multidrug resistance phenotype even for drugs not directly transported by ABCB1. A detailed characterization of the potential transport of membrane lipid derivatives by ABCG2 has not been reported as yet.

3. EFFECTS OF LIPIDS ON THE EXPRESSION OF ABCB1 AND ABCG2: REGULATION BY NUCLEAR RECEPTORS

As discussed in the previous sections, lipids can significantly modify the substrate handling, the ATPase activity, and the transport function of the ABCB1 and ABCG2 proteins. In addition, an increasing body of evidence suggests that cellular lipid species might also influence the expression of these MDR-ABC transporters by regulating their gene expression via nuclear receptors (NRs).

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s0045
3.1. The NR superfamily of transcription factors and lipid-sensing NRs

p0270 The NR superfamily comprises 48 members in the human genome and represents the largest currently known family of transcription factors. NRs work as ligand-activated transcription factors and affect various cellular processes such as reproduction, development, and metabolism through regulating the expression of their target genes. Following the cloning of the first steroid hormone receptors, the glucocorticoid (GR) and estrogen (ER) receptors in the mid-1980s, early studies established the fundamental role of hormone–receptor complexes in transcriptional regulation and endocrine signaling and surprisingly also revealed the existence of several evolutionarily related proteins all sharing a common modular structure. The three functional domains shared by NRs are (i) the N-terminal ligand-independent transactivation domain which is recognized by coactivators and/or other transcription factors; (ii) the most highly conserved central DNA-binding domain with two zinc-finger motifs that besides binding to DNA is also important in NR dimerization; and (iii) the C-terminal ligand-binding domain which also displays a ligand-induced activation function involved in transcriptional coregulator interaction. The basic mechanism of transcriptional regulation of the target genes is also shared among the NR family members: upon ligand binding, the typically cytosolic resident NRs dissociate from their partner proteins, shuttle to the nucleus, and bind to the NR response elements (derivatives of the canonical sequence RGGTCA termed as hormone response element where R stands for purine) of the target genes as monomers, homodimers, or heterodimers, and by recruiting various coactivator or corepressor complexes modulate transcriptional activity (Chan, Hoque, & Bendayan, 2013; Evans, 2005; Evans & Mangelsdorf, 2014; Gronemeyer, Gustafsson, & Laudet, 2004).

p0275 Despite the structural similarities, the ligand sensitivity of the different NRs shows wide variations. According to the ligand-binding specificities, NRs can be divided into three classes: (i) steroid and endocrine receptors, (ii) true orphan receptors, and (iii) adopted orphan receptors. Steroid and endocrine NRs, such as the estrogen receptor (ER) or the progesterone receptor (PR), were identified through analyzing the effect of known hormones, and this class of NRs represents important mediators of endocrine signaling. In contrast, orphan receptors have been identified through molecular sequence analysis without any prior knowledge about their ligand spectrum, and while the ligands of true orphan receptors are still to be identified,

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adopted orphan NRs have recently been found to be involved in xenobiotic and in lipid sensing (Chan et al., 2013; Chawla, Repa, Evans, & Mangelsdorf, 2001; Handschin & Meyer, 2005; Klaassen & Aleksunes, 2010; Wang & LeCluyse, 2003).

p0280 The lipid-sensing adopted orphan NRs include the sterol sensor LXR (liver X receptor), the bile acid sensor FXR (farnesoid X receptor), the fatty acid sensor PPAR (peroxisome proliferator-activated receptor). The xenosensors CAR (constitutive androstane receptor) and PXR/SXR (pregnane X receptor/steroid xenobiotic receptor) can also interact with lipid ligands (Chawla et al., 2001; Evans & Mangelsdorf, 2014; Handschin & Meyer, 2005; Ory, 2004). These NRs must heterodimerize with RXR (retinoid X receptor) to exert their gene regulatory function. The aforementioned NRs have been reported to be expressed in various tissues, including the intestine, the liver, and the central nervous system, and notably show an overlapping tissue expression pattern with several ABC transporters, including ABCB1 and ABCG2 (Chan et al., 2013; Jonker, Stedman, Liddle, & Downes, 2009).

p0285 Lipid- and xenobiotic-sensing NRs have been implicated in the orchestrated transcriptional regulation of enzymes and transporters involved in lipid metabolism or detoxification and thus their action provides a coordinated cellular response to perturbations affecting these processes (Fig. 3) (Chan et al., 2013; Chawla et al., 2001; Handschin & Meyer, 2005; Jonker et al., 2009; Ory, 2004; Urquhart, Tirona, & Kim, 2007). Professional lipid ABC transporters, such as the cholesterol and phospholipid transporter ABCA1, the long-chain PC and cholesterol transporter ABCB4 (MDR3), the bile salt transporter ABCB11 (BSEP/S-P-gp), or the major sterol transporters ABCG5/G8 (working as an obligate heterodimer), have indeed been shown to be regulated by lipid-sensing NRs (Beyea et al., 2007; Jonker et al., 2009; Schmitz & Langmann, 2005; Tarling et al., 2013; van Meer et al., 2008). Interestingly, to date, several NRs also involved in the recognition of lipid ligands have been shown to affect the gene expression of human ABCB1 and ABCG2, the transport functions of which are mainly involved in causing multidrug resistance (Borst & Elferink, 2002; Jonker et al., 2009; Klaassen & Aleksunes, 2010; Natarajan, Xie, Baer, & Ross, 2012; Sarkadi et al., 2006; Scotto, 2003). Therefore, via binding to and activating their cognate NRs, lipid molecules might also be directly involved in the transcriptional regulation of both ABCB1 and ABCG2.

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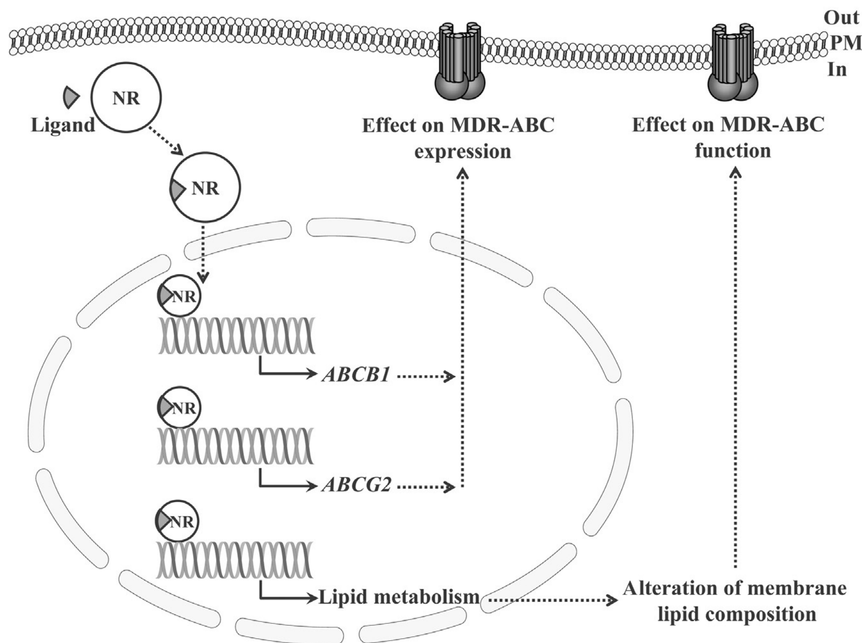

f0015 **Figure 3** *Regulation of MDR-ABC transporters by nuclear receptors (NRs)*. Upon binding their ligands, typically cytosol-resident NRs dissociate from their cytosolic partner proteins (not shown) and translocate to the nucleus where they heterodimerize with RXR (not shown), recruit various coactivators or corepressors (not shown), and modulate gene transcription. PXR, CAR, and PPARα and PPARγ response elements have been identified in the *ABCB1* and *ABCG2* gene promoters (see text for details). NRs have also been implicated in the regulation of lipid metabolism; therefore, besides direct transcriptional regulation of ABCB1 and ABCG2, by altering the membrane lipid composition, they might also be involved in the regulation of MDR-ABC transporter function. NR, nuclear receptor; PM, plasma membrane.

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s0050 3.2. Regulation of the expression of ABCB1 by NRs

p0290 Several NR response elements have been identified in the promoter of the ABCB1 gene. The 5′-upstream enhancer region of ABCB1 was shown to contain functional PXR- and CAR-binding sites. Upon ligand binding, both PXR and CAR were reported to induce mRNA expression of the transporter (Burk, Arnold, Geick, Tegude, & Eichelbaum, 2005; Cerveny et al., 2007; Geick, Eichelbaum, & Burk, 2001; Saeki, Kurose, Hasegawa, & Tohkin, 2011; Synold, Dussault, & Forman, 2001). Regulation of ABCB1 gene expression at the mRNA level was also suggested to occur by the PXR and CAR pathways in cultures of primary human hepatocytes (Jigorel, Le Vee, Boursier-Neyret, Parmentier, & Fardel, 2006;

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Maglich et al., 2002) and also at the human blood–brain barrier (Chan, Hoque, Cummins, & Bendayan, 2011). In contrast, in the human hepatocyte model cell HepG2, and also in rat liver, the lack of involvement of LXR in the regulation of ABCB1 expression was reported (Chisaki, Kobayashi, Itagaki, Hirano, & Iseki, 2009).

p0295 It has recently been shown that two other NRs, the thyroid hormone receptor β (TRβ) and the vitamin D receptor (VDR), also regulate the transcription of ABCB1 via binding as heterodimers with RXRα to the previously identified 5′-upstream enhancer region in the ABCB1 gene promoter (Kurose, Saeki, Tohkin, & Hasegawa, 2008; Saeki, Kurose, Tohkin, & Hasegawa, 2008).

s0055 3.3. Regulation of the expression of ABCG2 by NRs

p0300 The promoter of ABCG2 has been reported to contain an overlapping estrogen response element and a progesterone response element, which were shown to specifically bind ERα and progesterone receptor A and B (PRA and PRB), respectively. Expression of ABCG2 was reported to be induced by estrogen through ERα; however, enhanced expression of ABCG2 by progesterone was observed only in model cells expressing PRB, whereas PRA was shown to repress PRB activity (Ee et al., 2004; Wang et al., 2008).

p0305 Three functional PPAR response elements were also identified in the upstream enhancer region of ABCG2, and binding of the PPARγ–RXR heterodimer to this region was reported to be involved in the direct transcriptional upregulation of ABCG2 in human dendritic cells (Szatmari et al., 2006) (Fig. 3B). Recently, another isoform, PPARα, has also been shown to directly induce ABCG2 mRNA and protein expression through binding to the same conserved enhancer region of the ABCG2 gene in human brain microvessel endothelial cells (Hoque, Robillard, & Bendayan, 2012).

p0310 In primary human hepatocytes, exposure to certain NR ligands resulted in enhanced ABCG2 mRNA expression, strongly suggesting the regulatory role of the PXR and CAR pathways (Jigorel et al., 2006). In a following study, a CAR/RXRα-binding motif in the distal promoter of ABCG2 was identified and, interestingly, ABCG2 transactivation through this motif was found to occur via CAR but not PXR binding (Benoki, Yoshinari, Chikada, Imai, & Yamazoe, 2012). In contrast, it has been reported that LXR agonists do not activate the transcription of ABCG2 either in the

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human hepatocyte cell line HepG2 *in vitro* or in the rat liver *in vivo* (Chisaki et al., 2009).

3.4. Role of NRs in lipid metabolism and a potential indirect effect on ABCB1 and ABCG2 transporter function

As mentioned above, NRs participate in the coordinated regulation of transcriptional programs which can influence overall lipid metabolism (Chawla et al., 2001; Handschin & Meyer, 2005; Ory, 2004). As detailed in the sections above, function of the membrane MDR-ABC transporters can significantly be modified by their membrane lipid environment. Therefore, it is interesting to note that besides direct regulation of the MDR-ABC gene transcription, NRs might also exert an indirect regulatory effect on MDR-ABC function by modulating the levels of lipid species, such as cholesterol, which are important in maintaining the structures of biomembranes. According to a recent study, LXR signaling is indeed involved in the dynamic modulation of membrane lipid composition by promoting the incorporation of unsaturated fatty acids into phospholipids in response to changes in cellular lipid metabolism. This LXR-mediated phospholipid remodeling might affect the biophysical characteristics of biological membranes and thus might also be involved in the modification of transmembrane protein function (Rong et al., 2013).

4. EXPERIMENTAL STRATEGIES TO DEFINE THE LIPID-INTERACTING REGIONS OF THE ABCB1 AND ABCG2 PROTEINS

As discussed in Section 2, the activity of ABCB1 and ABCG2 is tightly regulated by their lipid environment, and their function is practically inseparable from the presence of surrounding lipids. Still, the exact nature of this complex protein–lipid interaction is not fully understood. Furthermore, protein regions of ABCB1 and ABCG2 responsible for direct interaction with lipids are not properly mapped, although in recent years numerous studies have dealt with this question. The main strategies applied in order to identify the lipid "sensor" regions include site directed mutagenesis of the transporters, measurements of direct binding of lipids to the proteins, and molecular dynamics (MD) simulations. In this section, we summarize the data regarding lipid-sensing regions of ABCB1 and ABCG2 determined by these techniques.

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p0325 One of the strategies to explore the potential interaction of predicted protein motifs with selected lipids is the alteration of amino acids of the given region by *site-directed mutagenesis*. One drawback of this method is that finding the lipid-binding consensus sequences defined by linear peptide sequences in large proteins is inherently difficult. Still, a systematic, non-biased mutagenesis approach may circumvent this problem (see below and Loo, Bartlett, & Clarke, 2009), and also the atomic level structural data will greatly promote the determination of the lipid-sensing structural patterns. The other limitation of the site directed mutagenesis-based approach is the difficulty to distinguish between a mutation directly inactivating the protein, e.g., altering the transport or ATP hydrolytic activity and one selectively altering the cholesterol/lipid sensitivity of the transporter. This uncertainty is caused by the fact that the functions of ABCB1 and ABCG2 are inseparable from their lipid environment, and there are practically no assays capable of distinguishing between the aforementioned two phenotypes. *Direct binding studies* measuring the interaction between lipids and ABC proteins may provide an independent approach to avoid these problems. However, as lipids tend to bind to hydrophobic surfaces due to their hydrophobic nature, this approach necessitates purified ABC proteins and well-chosen controls to separate specific lipid binding from simple hydrophobic interactions ("sticking"). A detailed description of structural studies-based *molecular simulations* for examining MDR-ABC transporter and lipid interactions is discussed in Section 5.

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s0070 4.1. Lipid sensing by the ABCB1 protein

s0075 *4.1.1 Mutagenesis studies in ABCB1*

p0330 The lipid sensing of ABCB1 has been extensively investigated by mutagenesis studies targeting potential amino acids involved in ABCB1–lipid interactions. Since ABCB1 is involved in the transport of lipids and lipid derivatives (see Section 2), some lipids and related substrates or inhibitors most probably share the substrate-binding pocket. On the other hand, lipids, which display modulatory effects and alter function and localization independently from substrate binding, most probably interact with the transporter at a different binding site defined by membrane facing amino acids. Here, we summarize the known lipid-sensing positions in ABCB1 identified by site-directed mutagenesis demonstrating both types of interactions.

p0335 It has been shown that AdaGb3 (adamantyl globotriaosylceramide), a water-soluble ganglioside analogue, inhibits ABCB1-mediated vinblastine

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efflux, indicating that the lipid-binding site interferes with substrate binding (De Rosa et al., 2008). ABCB1 partially colocalizes with globotriaosylceramide at the cell surface, and glycosphingolipid depletion results in a decreased cell surface expression of ABCB1, indicating that gangliosides also influence the trafficking of this protein. In order to identify the positions of the large substrate-binding pocket influencing the binding of this globotriaosylceramide analogue, De Rosa et al. (2008) introduced cysteine pairs into several positions (F343C in TM6, in combination with F728C in TM7, or Q725C in TM7; and L339C in TM6 with F728C in TM7) previously demonstrated to be involved in substrate binding (Loo, Bartlett, & Clarke, 2006). If cross-linking could not be observed between the two cysteines in the presence of the lipid, then lipid binding at least to one of the specific amino acid positions was concluded. By applying this strategy, the authors identified L339C in TM6 to be responsible for AdaGb3 binding.

p0340 With regard to amino acids interacting with the lipid environment but not involved in direct lipid transport, more extensive data are available. Loo and Clarke performed numerous studies in order to define amino acids facing the membrane phase, thus interacting with the lipid environment. In these studies, a construct with the G251V mutation, causing protein instability and improper maturation (processing mutant), was used in combination with Arg mutations in transmembrane helices (Loo, Bartlett, & Clarke, 2008; Loo et al., 2009; Loo & Clarke, 2013). As a result, some "corrector" mutations rescued the processing mutant G251V by promoting its proper folding by Loo et al. (2008). The authors hypothesized that when the inserted Arg faces the lipid interface, the rescue fails because of the intolerability of the positive charge in the hydrophobic environment (see Fig. 4). Based on the same rationale, Loo and Clarke also performed a large scale mutagenesis study in which arginines were introduced along all potential transmembrane helices in ABCB1 (Loo et al., 2009). By using the same technique, Loo and Clarke also replaced all amino acids found in TM helices 5 and 9. Most of the amino acids identified in their study fit well to the 3D model built based on the crystal structure of Sav1866; namely these side chains are looking toward the membrane phase (see Fig. 4). However, some of the positions identified as lipid-interacting amino acids direct their side chains toward the substrate-binding cavity and not toward the bilayer (e.g., in TM10 and 11). One possible explanation for this is that data obtained from the crystal structure represent only a snapshot; however during the catalytic cycle, the protein undergoes major conformational changes

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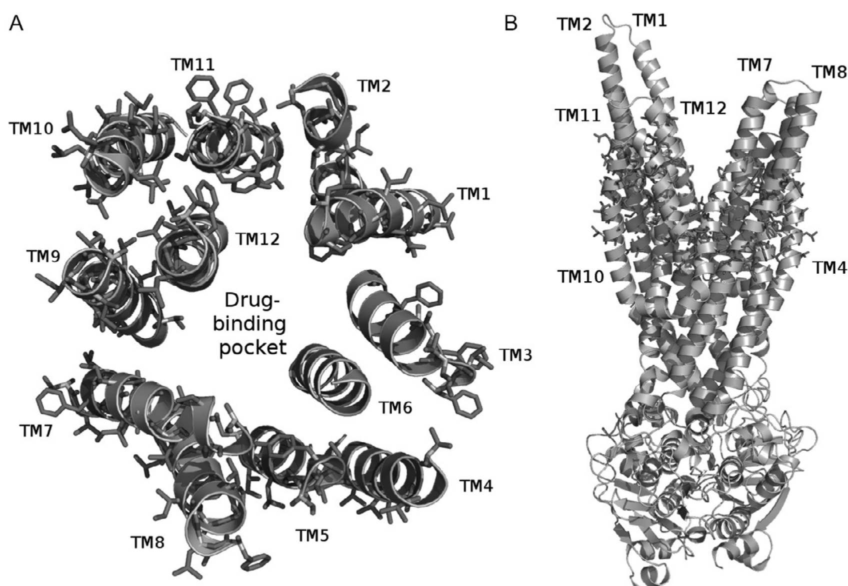

f0020 **Figure 4** *Amino acids in ABCB1, facing the lipid interface.* The upper view (A) and side view (B) of the model of the ABCB1 protein created based on the structure of Sav1866 (Globisch, Pajeva, & Wiese, 2008). Lipid-facing amino acids identified by mutagenesis studies are indicated by red color (for details see text). *Figure is based on Loo et al. (2009).*

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during which the side chains of the lipid interacting amino acid can face the membrane phase.

p0345 Recently, relatively detailed atomic level crystal structures have become available for the homologues of human ABCB1, the *Caenorhabditis elegans*, and the mouse Abcb1 proteins (Aller et al., 2009; Jin, Oldham, Zhang, & Chen, 2012). Thus, proper localization of the amino acids within and outside the transmembrane helices can be estimated. Therefore, it can be expected that these structural data will greatly promote the assignment of additional lipid-sensing amino acid positions that can be further verified by site-directed mutagenesis studies.

s0080 *4.1.2 Direct binding of lipids and MD simulations on ABCB1*

p0350 As discussed above, measurement of lipid binding to the ABCB1 protein can provide direct data about the protein regions involved in lipid interaction independently of the substrate transport function. Moreover, lipids interacting with ABCB1 can also be identified by combining lipid binding with

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mass spectrometry as demonstrated by Marcoux et al. (2013). They used purified mouse Abcb1 in a detergent (*n*-dodecyl-β-D-maltoside—DDM) containing buffer and analyzed direct lipid binding to Abcb1 and then determined the bound lipids by mass spectrometry. In this study, three cardiolipin molecules were found bound to the substrate-binding cavity of Abcb1, and by related calculations, the size and number of lipids present simultaneously in the substrate-binding pocket were also determined (Marcoux et al., 2013). As a further expansion of this study, Marcoux et al. performed molecular docking in which they showed that cardiolipin-14 binds to K230 and K822 found in transmembrane helices TM4 and 9 previously shown to be involved in lipid sensing (see Section 4.1.1).

p0355 The other type of lipids investigated for direct binding to ABCB1 was cholesterol. Kimura, Kioka, et al. (2007) have shown that cholesterol can directly bind to purified ABCB1. Additionally, they have systematically analyzed the effect of cholesterol on the ATP hydrolytic activity of ABCB1. Cholesterol was observed to activate ATP hydrolysis differently, depending on the size of the investigated substrate as we discussed earlier. In the case of small transported molecules (molecular mass below 800 Da), cholesterol stimulated the ABCB1-ATPase, while cholesterol had no effect on the ATPase activity in the presence of larger substrates. In the former case, cholesterol altered the substrate K_M values, indicating that this lipid directly interacts with the substrate-binding sites. Based on the alternative effects of cholesterol on ABCB1 function, the authors devised a cholesterol fill-in model (see Fig. 5A and B and Kimura, Kodan, et al., 2007), suggesting that in the case of smaller transported substrates (Fig. 5A), cholesterol can be present in the cavity simultaneously with the substrate and can promote the entrance and/or exit of substrate molecules, while in the case of larger substrates there is no space for cholesterol (Fig. 5B). Later, in the crystal structure of *C. elegans* Abcb1 two molecules of a lipid-like substance (*n*-undecyl-β-maltoside) were found to be located inside the drug-binding cavity (Jin et al., 2012) confirming that lipids can enter the substrate-binding pocket.

p0360 Besides experimental data, *in silico* modeling can also be used to determine protein–lipid interactions. In the case of ABCB1, Wen, Verhalen, Wilkens, McHaourab, and Tajkhorshid (2013) performed MD simulations. According to their results, the lipid enters between TM3, 4, and 6 and interacts with Leu-300, Ala-302, Tyr-303, and Ala-338, previously shown to be involved in drug binding (for details see Section 5).

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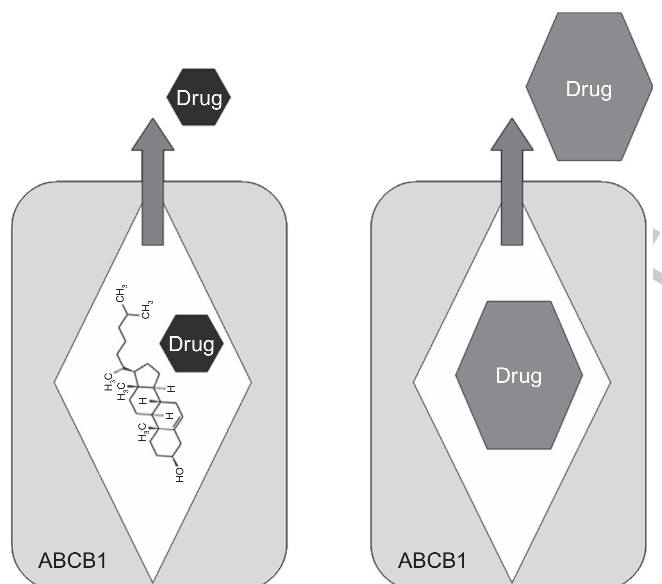

f0025 **Figure 5** *Cholesterol fill-in model for ABCB1 function*. In the case of small compounds (A), cholesterol promotes the transport by filling the substrate-binding pocket, while in the case of larger substrates (B), the compound itself fills the cavity. *Figure is based on the model of Kimura, Kodan, et al. (2007).*

s0085 4.2. Lipid sensing by the ABCG2 protein

p0365 In the case of ABCG2, literature for its interaction with lipids is far more incomplete than in the case of ABCB1. Since no atomic level crystal structure is available for any closely related ABCG-type proteins, the localization of amino acids in the transmembrane helices cannot be properly estimated. The homology models constructed on the basis of unrelated ABC transporters (Hazai & Bikadi, 2008; Li et al., 2007) are contradictory and cannot be properly applied for devising site-directed mutagenesis.

p0370 Still, information obtained by site-directed mutagenesis and related to potential lipid sensors and lipid-binding sites in ABCG2 is already available regarding the interaction of cholesterol and some sterol compounds with the transporter. As described in Section 2, cholesterol and bile acids have significant roles in ABCG2 function: ABCG2 is practically inactive in the absence of cholesterol, while bile acids selectively modulate the basal ATPase activity of the transporter. In this section, we list the amino acid regions examined in detail for such sterol interactions.

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4.2.1 Role of the R482 position

It turned out very early, practically at the cloning of ABCG2 that R482 is a crucial determinant of substrate recognition of ABCG2. In these early studies, it was found that a mutation of R482 to G or T occurred in the cell lines upon anthracycline selection, most probably because these "artificial" (not occurring *in vivo)* mutant ABCG2 variants results in a "gain-of-function" transporter with increased doxorubicin and rhodamine 123 transport activity (Chen et al., 2003; Honjo et al., 2001; Miwa et al., 2003; Ozvegy, Varadi, & Sarkadi, 2002). Later it turned out that amino acid 482 is also involved in the cholesterol and bile acid sensing of the protein. Interestingly, Telbisz et al. (2007) found that in contrast to the wild-type protein, the R482G mutant is fully active in *Sf9* insect membrane vesicles containing only low levels of cholesterol (see Section 2). Therefore, ABCG2-R482G seemed to function in a cholesterol-independent way. Surprisingly, when the isolated, purified, and reconstituted ABCG2-R482G mutant was characterized in proteoliposomes, providing a well-controlled lipid environment, it was found that this mutant also required cholesterol for its activity. Moreover, the mutant variant had an increased cholesterol affinity, that is the mutant variant was fully active at much lower cholesterol concentrations in the proteoliposomes than that required for the wild-type protein (see Fig. 6A and Telbisz et al., 2013). This finding explained the apparent cholesterol insensitivity of the R482 mutant in the *Sf9* membranes, which contain only low level of sterols that were already sufficient to provide full ABCG2 activity.

Recently, amino acid position 482 was further analyzed in detail with regard to its influence on the cholesterol-sensing of ABCG2 (Telbisz et al., 2014). In their study, Telbisz et al. found that replacement of R482 to smaller amino acids (D, N, S, and T) resulted in a cholesterol-independent behavior of these mutant proteins in *Sf9* insect membranes. In contrast, larger size amino acids (I, K, M, and Y) produced similar cholesterol sensitivity as the wild-type protein. However and surprisingly, the wild-type protein proved to be unique in regard with its modulation by bile acids. All R482 mutants, even those with larger amino acid side chains, behaved differently from the wild-type transporter (Fig. 6B).

4.2.2 Role of the LxxL motif

In several sterol-binding proteins (e.g., progesterone, androgen, estrogen, or glucocorticoid receptors and oxysterol-binding protein-related proteins), an LxxLxxL motif (x standing for any amino acid) was identified as a steroid-binding element (Im, Raychaudhuri, Prinz, & Hurley, 2005;

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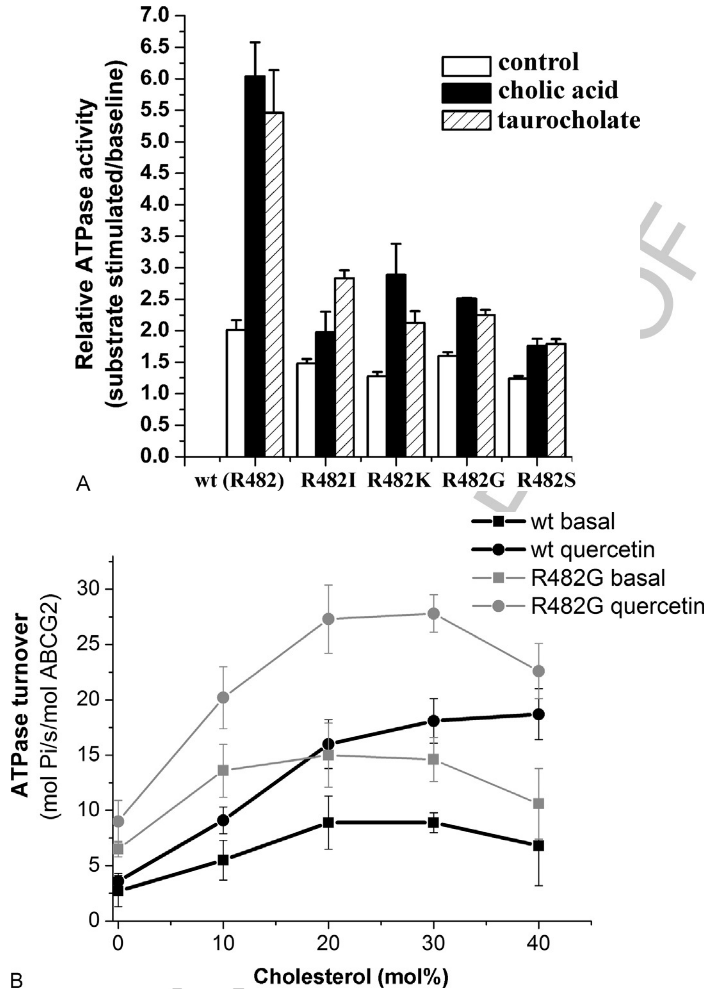

f0030 **Figure 6** *Different positions involved in the sterol-sensing of ABCG2*. (A) The R482G mutant is fully active at lower cholesterol levels. ATPase activity measurement in purified ABCG2 (wild type and the R482G mutant) reconstituted in proteoliposomes provides an excellent tool to investigate ABCG2 activity in a well-controlled lipid environment. This experiment shows that in the case of the R482G mutant, both the basal and the substrate (quercetin)-stimulated ATPase activities are more sensitive to cholesterol than those of the wild-type protein (Telbisz et al., 2013). (B) The bile-acid modulation of wild-type ABCG2 is unique. In *Sf9* insect membranes containing wtABCG2 (R482), the relative substrate stimulation of the ATPase activity is significantly higher in the presence of bile acids. When R482 is mutated, the effect of bile acids is greatly decreased.

Au8

(Continued)

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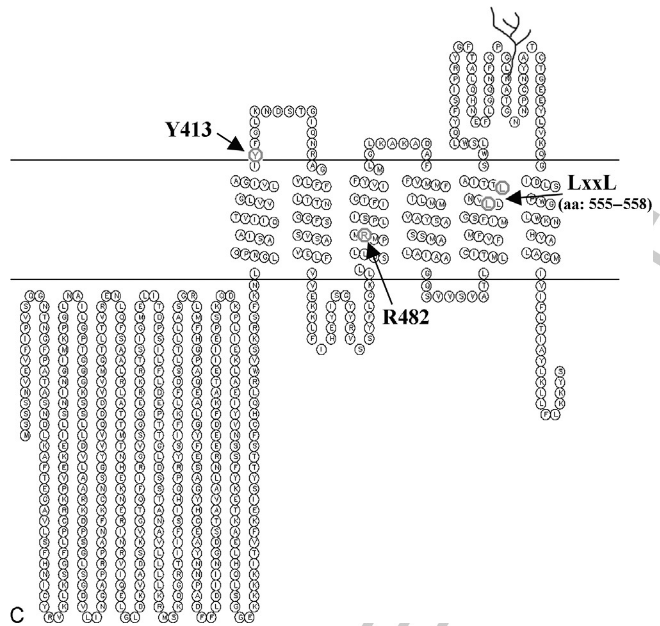

f0030 **Figure 6—Cont'd** (C) Positions of amino acids involved in sterol sensing of ABCG2. The topology of ABCG2 has been determined using HMMTOP (Tusnady & Simon, 2001) and drawn by using http://emboss.bioinformatics.nl/cgi-bin/emboss/topo. Amino acids involved in lipid sensing are indicated. *Panel (B): Adapted from Telbisz et al. (2014).*

Williams & Sigler, 1998). Although this is a relatively nonconserved sequence, in ABCG2, only a shorter LxxL motif (amino acids 555–558) can be found (Fig. 6C), which has been examined in detail as a potential lipid interactive or sensor region.

p0390 Velamakanni, Janvilisri, Shahi, and van Veen (2008) by expressing ABCG2 in *L. lactis* found that the L555A/L558A mutant loses its progesterone and estradiol recognition capacity. In another study, Telbisz et al. (2014) observed that in *Sf9* membranes the L555A and L558A mutants behaved in a cholesterol-independent manner. However, in proteoliposomes, the L558A mutant also required cholesterol for its ATPase activity. Unfortunately, the L555A/L558A mutant could not be investigated in its purified and reconstituted form because an inactivation due to loss of dimerization occurred during purification. This latter observation may indicate that cholesterol promotes proper membrane insertion and the formation of the ABCG2 homodimer, and the double mutant becomes nonfunctional because of the loss of lipid sensing in this protein region.

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s0100 ### 4.2.3 Role of the CRAC motif

p0395 Several conserved motifs have already been experimentally shown to be involved in cholesterol sensing in "professional" cholesterol-binding proteins. These motifs include the sterol-sensing domain comprising five transmembrane helices, the YIYF sequence, the CRAC (cholesterol recognition amino acid consensus), and the CARC ("inverted" CRAC) motifs (Baier, Fantini, & Barrantes, 2011; Epand, 2006). In the case of ABCG2, the potential role of the CRAC motif (L/V-X_{1-5}-Y-X_{1-5}-R/K, X standing for any amino acid) has recently been analyzed in detail by site-directed mutagenesis (Gál et al., 2014). In this work, the central, conserved tyrosines were mutated in five potential CRAC motifs in ABCG2, and the effects of single mutants on the cholesterol-sensing capability of ABCG2 were analyzed. Interestingly, it was found that three of the five positions analyzed were important in proper protein folding, while Y413 found in the predicted TM helix 1 (Fig. 6C) caused increased cholesterol sensitivity of the protein.

p0400 As a conclusion, mutagenesis studies performed on ABCG2 provided variable results, and in some cases, the opposite effect was observed to what was expected. Both the R482G and the Y413S mutants which were expected to eliminate sterol binding actually increased the apparent affinity of the transporter for cholesterol. The experimental examination of the CRAC motifs also provided only incomplete information; thus, additional studies are required to map the lipid interacting parts of ABCG2. Until now, no direct lipid-binding experiments have been performed in the case of this transporter.

s0105 ## 5. *IN SILICO* MODELING OF THE LIPID INTERACTIONS OF ABCB1 AND ABCG2

p0405 Experimental atomic level exploration of the lipid–protein interactions is challenging. Various spectroscopic methods, including NMR and ESR, have been employed to study these interactions, but provided information mostly on average orientational order (Marsh & Pali, 2013). Crystal structures of membrane proteins obtained by X-ray crystallography in numerous cases contain associated lipid molecules. However, the orientation and conformation of the lipids in the crystals are indicated to be different from that in a lipid bilayer (Marsh & Pali, 2013). As alternative methodologies, computational approaches can be applied to describe lipid–protein interactions at the atomic level.

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p0410 All the computational methods discussed here, such as MD simulation or *in silico* docking, require high-resolution 3D structure of the protein under investigation. While murine Abcb1 has been crystallized in an apo state (Aller et al., 2009), and sufficient homology models can be built for its ATP-bound conformation (Globisch et al., 2008; O'Mara & Tieleman, 2007; Pajeva, Globisch, & Wiese, 2009), structural or homology models of human ABCG2 are insufficient for *in silico* studies because of the low-resolution structure information (cryoelectron microscopy, >5 Å) (Rosenberg et al., 2010) and the very low (<20%) sequence similarity of the TMD with any existing ABC structure that could be applied as a possible template.

s0110 5.1. MD simulation

p0415 MD computer simulations attempt to describe the time-dependent movements and interactions of the atoms in the examined protein and its close environment by numerically solving the equations of molecular mechanics force fields. Thus, MD simulations for a transmembrane protein in a lipid bilayer may be suitable to describe conformational changes within the protein and the movements of the lipids around the protein (Tieleman et al., 2006). When a lipid molecule interacts with a specific region of the protein, it "sticks" there and usually interacts with specific amino acids for the remaining part of the simulation. However, the timescale of the MD simulations with a large system containing all atoms of all relevant molecules is highly limited, and the association of a lipid with a specific region of the protein is a relatively rare event.

p0420 Wen et al. (2013) have performed MD simulations using the mouse Abcb1 (P-gp) structure (PDB:3G5U) in the apo conformation, with the NBDs apart. Since the dynamics and lipid interactions in the simulations may be affected by the initial lipid contacts of the protein, they have generated four different systems with the protein differently embedded in a bilayer built from POPE, for parallel simulations. In the case of one of the four systems, in 50-ns long simulations, the protrusion of a lipid molecule into the cleft between TM4 and 6 could be observed. These TM helices delineate the putative drug entry site into the central cavity of the protein (Aller et al., 2009). As ABCB1 has been indicated to possess lipid transport activity and its drug substrates are hydrophobic, this protrusion event has been suggested to be equivalent with the initial steps of drug binding.

p0425 To overcome the limitation of the simulation time scale, coarse-grained (CG) models can be applied to simplify the system under investigation (Ding, Buldyrev, & Dokholyan, 2005; Klein & Shinoda, 2008;

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Marrink & Tieleman, 2013). In the case of CG models, several atoms are represented by a single bead (virtual atom) in a way to preserve the topology and physicochemical properties of the coarse-grained molecule. As an example, the most widely used MARTINI model (Marrink & Tieleman, 2013), on average maps four heavy atoms and associated hydrogens to a single interaction center. In addition, four main interaction types are defined, such as apolar, nonpolar, polar, and charged. As an example, four water molecules can be mapped to one CG water bead, and one ion is mapped to a charged bead that also represents the first hydration shell. However, this type of mapping is insufficient for describing complex chemical entities, including ring-like structures. When a higher resolution mapping is applied, the force fields of the CG models to describe the interactions between the beads are parametrized in a systematic way: (1) nonbonded interactions are tuned to reproduce experimental partitioning free energies between polar and apolar phases of various chemical compounds, and (2) bonded interactions are defined and calculated by using reference all-atom simulations.

p0430 A prominent example to decipher lipid–protein interaction by employing CG MDs has been provided by Schmidt et al., studying the phosphatidylinositol 4,5-bisphosphate (PIP2) binding to the Kir2.2 inwardly rectifying potassium channel (Schmidt, Stansfeld, Tucker, & Sansom, 2013). At the first stage, they performed coarse-grained simulations with Kir2.2 and PIP2 placed in a distance from each other in a bilayer. These simulations and events at this level are sufficiently fast to observe PIP2 diffusion and association with the potassium channel. At the second stage, the simplified model of the associated complex and bilayer was converted to all-atom model for simulations with higher accuracy. Their results, when compared to experimental data, suggest that multiscale approaches involving coarse-grained models may properly describe protein–lipid interactions, thus could be also applied to investigate ABC protein and lipid interactions.

s0115 5.2. *In silico* docking

p0435 A different approach to study molecular interactions is *in silico* docking of small molecules to target proteins. In this case, the protein is usually handled as a rigid molecule, in contrast to the small molecule that is allowed to rotate at its bonds. However, docking of lipids is challenging because of their high rotational freedom (bonds of the hydrophobic chains), that impair the performance of most docking algorithms. Docking of ligands with high number of atoms is often prohibited even in commercial software. Moreover, the location of the docking, namely the volume of the search place, is also

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limited and requires *a priori* knowledge about the binding site. A recently developed free tool, Autodock Vina (Trott & Olson, 2010), can handle flexible molecules with high number of rotatable bonds and allows to perform docking in short time with no serious restrictions on putative binding locations.

p0440 In a recent study, binding of drugs and cardiolipins with different sizes to the ABCB1 protein has been investigated by using mass spectrometry and molecular docking (Marcoux et al., 2013). Importantly, the computational and experimental results were concordant, and the presence of two molecules of the smaller cardiolipin CL14 or only one molecule of the larger CL24 was indicated in the internal binding pocket of ABCB1. The docking of the ligands was performed in two steps: (1) simulation with Patchdock (Schneidman-Duhovny, Inbar, Nussinov, & Wolfson, 2005), employing a shape complementary criteria; (2) accurate refinement of the orientation of side chain and ligand atoms, using FireDock (Mashiach, Schneidman-Duhovny, Andrusier, Nussinov, & Wolfson, 2008).

p0445 As a summary, application of computational methods for studying lipid–protein interactions is relatively sparse, because of the limitation of the available tools and the experimental difficulties that make it challenging to correlate theoretical and experimental results. However, for resolving several questions, at the atomic level, currently only the *in silico* methods are available. Careful application of these approaches may provide valuable information on the effects of lipids on MDR-ABC protein stability and conformation.

s0120

6. CONCLUSIONS

p0450 ABCB1 and ABCG2 are two major plasma membrane ABC multidrug transporters (MDR-ABC) involved in cancer chemotherapy resistance; thus, the exploration of their mechanism of action has a major therapeutic consequence. Both ABCB1 and ABCG2 are significantly modulated by various lipid compounds, especially those residing in the plasma membrane in their close proximity. In this chapter, we discuss the complex interactions of ABCB1 and ABCG2 with a variety of lipid molecules, focusing on the role of cholesterol and cholesterol derivatives. We demonstrate that lipids may affect MDR-ABC function at the transcriptional level, mainly by interacting with NRs. We also discuss lipid regulation of the MDR-ABC transporters at the posttranslational level and explore the molecular details of the direct transporter–lipid interactions, the exploration of potential lipid-sensor domains. Interestingly, these MDR-ABC proteins

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may also directly influence the general membrane composition, by "flopping" membrane lipid constituents. The further application of experimental site-directed mutagenesis, detailed structural studies, and *in silico* modeling should allow a more detailed understanding of the lipid interactions with these medically important MDR-ABC transporters.

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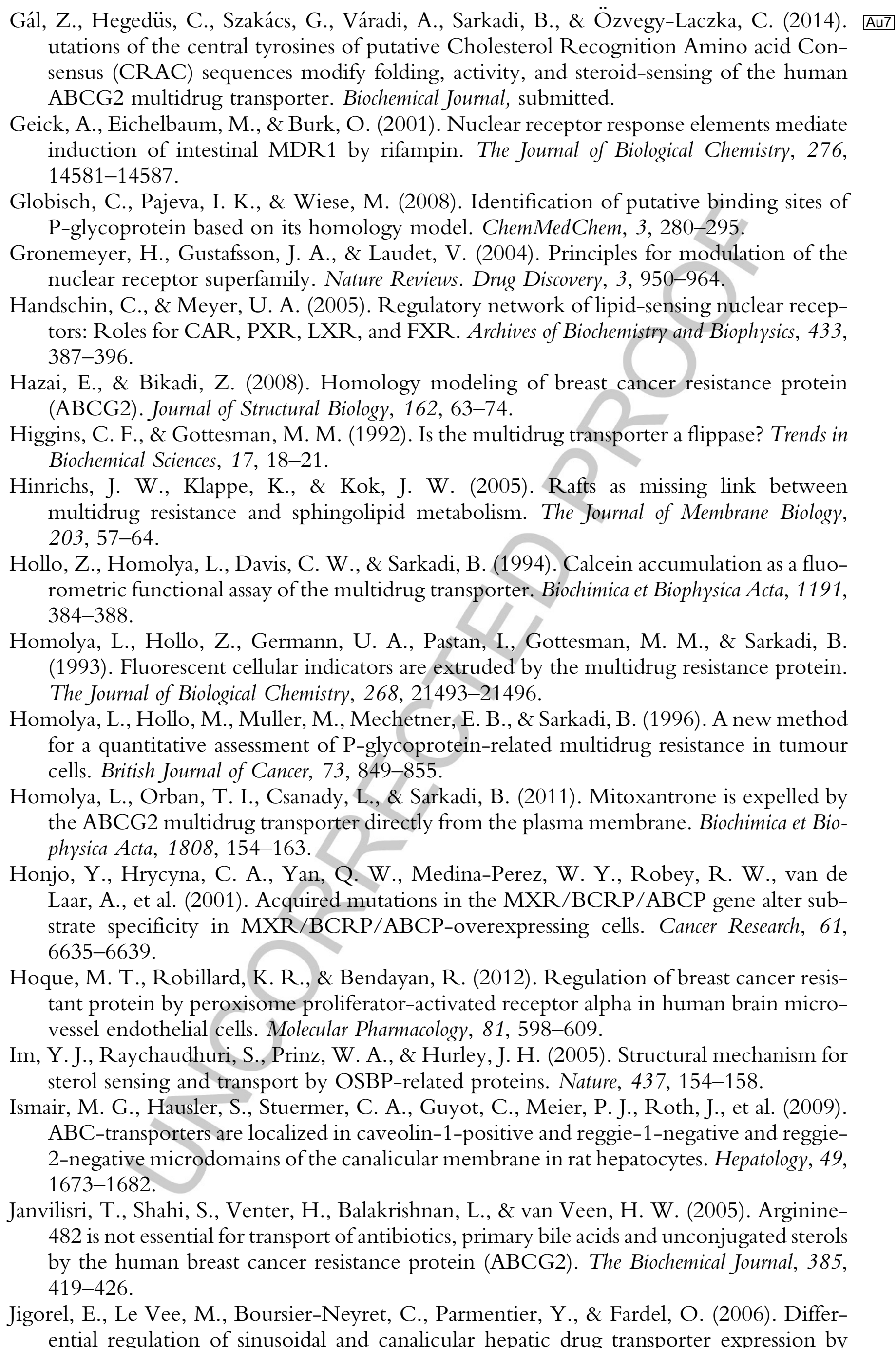

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Keywords: Membrane lipids, Cholesterol, Nuclear receptors, ABC multidrug transporter

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