

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Effects of polyphenols on P-glycoprotein (ABCB1) activity

by Kuljeet Singh

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND IMMUNE BIOLOGY

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1. List of Abbreviations

ABC: ATP-binding cassette

ABCB1: P-glycoprotein

ABCB11: Bile-salt export pump (BSEP)

ABCC1: multidrug resistance protein 1 (also called MRP1)

ABCG2: Breast Cancer Resistance Protein

ADME: Absorption, distribution, metabolism, elimination

AD: actinomycin D

AM: Acetoxymethylester

BSA: Bovine serum albumin

Calcein-AM: Calcein acetoxymethylester

Cryo-EM: Cryo-electron microscopy

CsA: Cyclosporine A

C3S: cyanidin-3O-sophoroside

DNR: daunorubicin

DMEM: Dulbecco's modified Eagle's medium

DPH: 1,6-Diphenyl-1,3,5-hexatriene

DTT: L-dithiothreitol

EPR: Electron Paramagnetic Resonance

Fab: Antigen-binding fragment

FCS: Fetal calf serum

IAAP: (¹²⁵I)-iodoarylazidoprazosin

ICH: intracellular coupling helix

ICL: intracellular loop

LogPow: Octanol-water partition coefficient

MDR: Multidrug resistance

MD: Molecular dynamics

MDR1: Gene coding human P-glycoprotein (ABCB1)

MMPBSA: Molecular Mechanics Poisson-Boltzmann Surface Area calculations

MX: mitoxantrone

NBD: Nucleotide-binding domain

NBS: Nucleotide-binding site

Pgp: P-glycoprotein (ABCB1)

PI: Propidium iodide

Pi: Inorganic phosphate

PMSF: Phenylmethanesulfonylfluoride

PXE: Pseudoxanthoma elasticum

SASA: Solvent-accessible surface area

QUR: quercetin

SDS: Sodium dodecyl sulphate

TMH: Transmembrane helix

TMA-DPH: (1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5 Hexatriene

TMD: Transmembrane domain

2. Introduction, literature review

2.1. General introduction

The human ABCB1 (also referred as P-glycoprotein (Pgp) or multidrug resistance protein (MDR1)) belongs to the large family of ABC proteins. It is an integral membrane protein involved in the ATP-driven export of numerous chemically unrelated substrates from cells through the plasma membrane [1]. It has extremely broad substrate spectrum involving lipophilic or amphiphilic molecules, such as xenobiotics [2], metabolic side products [3] and numerous chemotherapeutic drugs used for the treatment of various diseases [4]. As it is shown in **Figure 1.**, ABCB1 is typically expressed in tissues with pharmacological barrier functions, e.g., in the intestinal epithelium, in the blood-brain, blood-placenta and blood-testis barriers and in organs involved in the elimination of toxic compounds from the human body, such as liver and kidneys [3]. Stem cells as well as cancer cells and cancer stem cells can also protect themselves from toxic compounds by the use of ABC transporters including ABCB1 and ABCG2 [5,6].

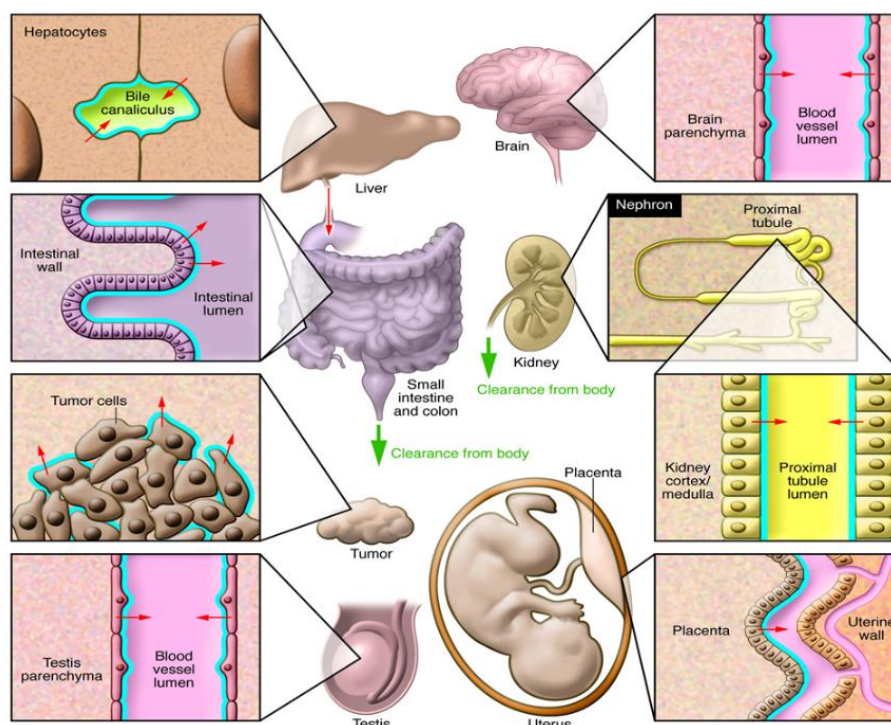


Figure 1. ABCB1 expression in the human body. Blue lines show the localization of ABCB1 and small red arrows indicate the direction of ABCB1 mediated transport based on [3].

Chemotherapy is an important therapeutic option for the treatment of malignancies, especially for systemic cancers, such as leukaemia, lymphoma and metastatic tumors [7]. Unfortunately, cancers often become resistant to numerous or sometimes practically all kinds

of anti-neoplastic agents that could be used in their elimination. The phenomenon, when tumor cells acquire cross-resistance against numerous chemotherapeutic agents is termed *multidrug resistance* (MDR) [8]. Since anticancer drugs often target proteins involved in cell cycle regulation [9], DNA repair [10] or apoptosis [11], alterations of these mechanisms may result in chemotherapy resistance [12]. Decreased effective concentration of the drug(s) at their target site(s) in tumor cells can also lead to failure of chemotherapy [13,14]. The major cause of the diminished cellular accumulation of drugs is the over-expression of certain ABC transporters (e.g., ABCB1, ABCG2 and ABCC1) in the plasma membrane of tumor cells.

Cancer is the second leading cause of death worldwide, and MDR is considered to be responsible for nearly 50% of ineffective tumor chemotherapies, and this fraction is close to 90% in case of metastatic cancers [15]. Therefore, the emergence of drug resistance is a serious impediment of successful chemotherapy. The detailed molecular level understanding of the ligand-ABC transporter interactions is of crucial importance, and it may help rational drug design to overcome cancer drug resistance[16].

2.2. Role of ABC transporters in multidrug resistance – a historical overview

Before the discovery and characterization of ABC transporters, several theories were formulated regarding the molecular basis of multidrug resistance in cancer cells. Over 50 years ago, Goldstein and colleagues described an actinomycin D (AD) resistant HeLa cell subline following intermittent exposure of cells to AD. Interestingly, resistant cells survived for long periods in culture media containing very high concentrations (1 to 3 µg/ml) of AD [17]. About 10 years later, June Biedler carried out experiments on several AD-resistant Chinese hamster lung and fibroblast cell lines maintaining them at high AD concentrations. Interestingly, these cells also exhibited up to 2500-fold resistance to vincristine and daunorubicin (DNR), as compared to AD untreated cells. On the other hand, several DNR-selected cell lines also exhibited increased resistance to AD. Collectively, these studies supported the hypothesis that the AD resistance of cells is due to changes of the cell membrane, resulting in decreased permeability to AD and certain other compounds [18].

Later on, Keld Dano [19] observed that DNR-resistant Ehrlich ascites tumour cells accumulated less DNR compared to wild type cells, while cell nuclei isolated from wild type and resistant cells exhibited similar DNR uptake, further supporting the hypothesis that the emergence of resistance is caused by changes of the plasma membrane permeability. The intracellular accumulation of DNR in resistant cells was increased by structural analogues of DNR (e.g., N-acetyl-DNR and daunorubicinol) and by metabolic inhibitors (such as 2-

deoxyglucose and iodoacetate) supporting that a specific active transport mechanism is responsible for the emergence of drug resistance. In addition, co-administration of certain chemically unrelated drugs, such as vincristine and vinblastine, also increased the intracellular accumulation of DNR in resistant cells supporting the competitive inhibition of the transport mechanism [20]. This active transporter was later identified in drug-resistant Chinese hamster ovary cells by Victor Ling and named as permeability glycoprotein (P-glycoprotein, Pgp), because its function seemed to be related to the permeability of the plasma membrane [21]. Later Gros and colleagues cloned the drug resistance gene from multidrug resistant Chinese hamster cells [22], and this gene was found to confer drug resistance when transfected into drug sensitive cells [23].

The cloning of the gene of human Pgp was also reported soon after in 1986 and it was named *MDR1* and later renamed *ABCB1* gene [24,25]. The expression level of ABCB1 was measured by western blot technique and it was found to positively correlate with the drug resistance of cancer cells [26]. In addition, it was also reported that transfection of sensitive cells with the *ABCB1* gene results in MDR phenotype [25,27]. Collectively, the above observations demonstrated that the expression of an active plasma membrane transporter with broad substrate spectrum can equip cells with the MDR phenotype. In the next decades all members of the human ABC protein superfamily were identified and few of them were proved to be multi-specific drug transporters, such as ABCB1, ABCG2 and ABCC1.

The human multidrug resistance-associated protein 1 (MRP1 or ABCC1) was identified by Cole and colleagues in 1992 [28], and was found to confer resistance to numerous cytotoxic drugs such as vincristine, vinblastine, paclitaxel, anthracyclines, mitoxantrone, flutamide and methotrexate. Since the majority of the above-mentioned compounds can be transported by ABCC1 in the form of glutathione, glucuronide, or sulphate conjugates typically formed upon phase II metabolism, ABCC1 is deemed to be an organic anion transporter [29]. Unfortunately, ABCC1 can also recognize and transport some new-generation anticancer agents that act by modifying components of different signalling pathways controlling the proliferation and metastatic potential of tumor cells [30].

The third clinically important multidrug transporter, ABCG2 (also known as Breast Cancer Resistance Protein [BCRP], Mitoxantrone Resistance Protein [MXR], and Placenta-Specific ABC transporter [ABCP]), was identified independently by three different groups within a period of few months [31,32]. Based on functional studies, ABCG2 mainly transports hydrophobic or amphipathic chemotherapeutic drugs [33]. Besides these compounds its

substrate spectrum also involves toxic metabolic side-products, such as pheophorbide A [34], estrone-3-sulfate [35] and uric acid [36].

2.3. General structure of ABC proteins

ABC proteins make up one of the largest super-families of proteins. They are present in all organisms from Archaea to humans [37]. In humans, 48 ABC proteins have been reported and these are divided into 7 distinct subfamilies from ABCA to ABCG, based on their sequence homology and structural similarities [38]. The majority of ABC proteins are active transporters, while some of them are ion-channels or ion-channel regulators. Interestingly, members of the E and F subfamilies do not possess transmembrane domains (TMDs) and they function as components of large enzyme complexes involved in mRNA splicing or transcription [38].

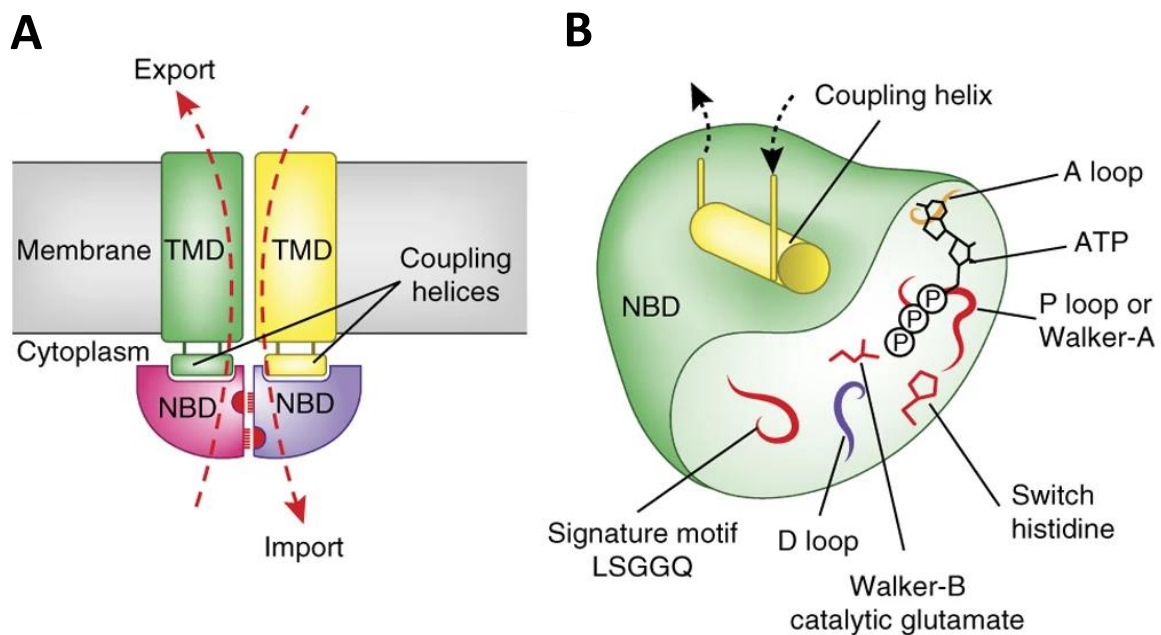


Figure 2. Domain arrangement **(A)** and the NBD interface of ABC transporters **(B)** At the NBD interface, red half-circles and dashed lines indicate the P loops and ABC signature motifs, respectively **(A)**. The evolutionary conserved sequence motifs important in ATP binding and hydrolysis are shown in panel **B** [39].

Functional ABC transporters have a characteristic four-domain architecture comprising of two evolutionary conserved cytoplasmic nucleotide-binding domains (NBDs), that bind and hydrolyse ATP, and two TMDs with diverse amino acid sequence, that recognize and transport a wide range of substrates (**Figure 2A**). The evolutionary conserved NBDs (**Figure 2B**) are built from two subdomains; the core or RecA-like subdomain, which contains the Walker A and B motifs and the α -helical domain, which involves the signature sequence (LSGGQ motif). The Walker A and Walker B sequences are present in almost all ATPases and many molecular

motors [40]. The signature sequence is unique to the NBDs of ABC transporters differentiating them from other ATP utilizing proteins [41]. In all ABC proteins, the conserved sequences of the NBDs form two composite catalytic sites, where the ATP molecules are sandwiched between the Walker A and Walker B motifs of one NBD and the signature sequence of the contralateral NBD [42]. In ABCB1 as well as in many other ABC transporters an important aromatic residue (called A-loop), usually a tyrosine (Y401 and Y1044 in ABCB1) interacts with the adenosine ring of the nucleotide and contributes to the strength of nucleotide binding [43, 44]. The Walker A motives bind the alpha and beta phosphates of ATP. The motif has the sequence G-x(4)-GK-[TS], where G, K, T and S denote glycine, lysine, threonine and serine residues respectively, and x denotes any amino acid. The lysine (K) residue in the Walker A motif is crucial for nucleotide-binding. The Walker B motives play a role in the coordination of Mg^{2+} , which is required for ATP hydrolysis. Neutralization of the acidic Walker B glutamate, frequently termed as the “catalytic glutamate”, in one or both NBSs, leads to high-affinity binding (trapping) of a single ATP molecule [45]. In certain ABC proteins the sequence of one of the ATP-binding sites (generally NBS1) differs from the consensus sequence and thus, it cannot hydrolyse ATP efficiently. For instance, the CFTR Cl^- -ion channel (ABCC7) and the channel regulator sulfonylurea receptor 1 and 2 (SUR1/2; ABCC8/9) have one degenerate ATP-binding site.

In contrast to the NBDs, the TMDs of ABC proteins show significant structural differences in accordance with their functional diversity. Bacteria, archaea, and fungi express both importer and exporter type ABC proteins with distinct and characteristic TMD folds, while humans and higher eukaryotes typically express exporters [45]. Importers mediate substrate transport toward the direction of their NBDs/cytosolic face of the membrane, while exporters transport their substrates away from their NBDs/cytosolic face of the membranes. Based on their general TMD structure (i.e., TMD fold) human ABC transporters belong to type I or type II ABC exporters [46]. In type I exporters (e.g., in ABCB1) each wing comprises TM helices from both halves of the transporter (**Figure 3A**). This structural feature is called “domain swapping”[47]. In contrast, type II ABC exporters (e.g., ABCG2) are not “domain swapped” [48] as it is shown in **Figure 3B**.

Moreover, certain ABC proteins also possess some accessory domains, such as the extracellular domain (ECD, see in **Figure 3B**) common to ABCA subfamily transporters [46] or the TMD0 domain found in certain ABCB and ABCC subfamily transporters, as it is shown in **Figure 3A** [46].

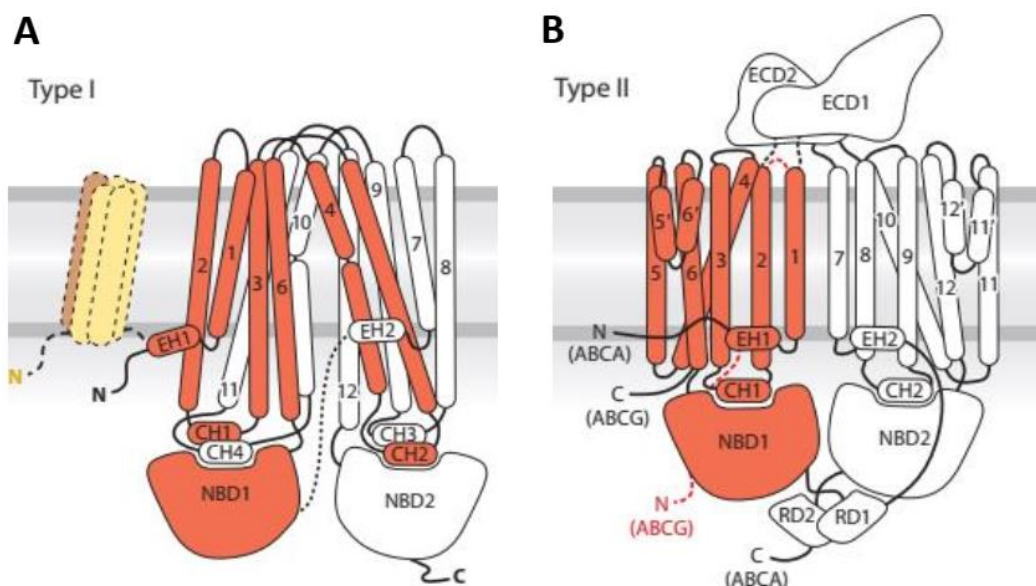


Figure 3. Typical topology of type I (A) and type II (B) ABC exporters. Membrane bilayer is depicted in grey. TMD0 is yellow, while the TMD1 and TMD2 of the transporters are represented in red and white, respectively (based on [46]).

2.4. Physiological functions and disorders associated with human ABC proteins

Several human ABC transporters are involved in the transport of lipophilic compounds, such as phospholipids, cholesterol and phytosterols through the plasma membrane or the membranes surrounding intracellular organelles. The ABCA subfamily member ABCA1 is involved in reverse cholesterol transport by exporting excess cholesterol and phospholipids from various cell types to lipid-poor apolipoproteins (apoA1 and apoE), which then form nascent high-density lipoproteins (HDL). Mutations decreasing the function or expression of ABCA1 reduce the level of high-density lipoprotein (HDL) in the blood and may cause Tangier-disease, a rare autosomal disorder [49]. Advancement of this disease results in accumulation of cholesterol-esters in peripheral reticuloendothelial tissues (e.g., lymph nodes, thymus and spleen) and is responsible for the early onset of cardiovascular diseases [50,51].

ABCA12, an another ABCA subfamily member, transports lipids including ceramides within the epidermal cells and it plays critical role in formation of the intercellular lipid layer, ultimately influencing the epidermal barrier function. Harlequin ichthyosis is due to inactivating mutations of ABCA12 [52]. The impaired barrier function of the skin causes difficulties in controlling water loss and regulating temperature [53]. In addition, the affected individuals are more susceptible to infections. Unfortunately, high fraction of the affected infants dies from microbial infections, feeding problems or respiratory distress [54]

ABCD1 mediates the transport of very-long chain fatty acids into peroxisomes for degradation. Mutations significantly reducing the expression or function of ABCD1 cause the fatal disorder adrenoleukodystrophy (ALD), which is characterized by the accumulation of very-long chain fatty acids in various tissues, such as in the central nervous system leading to demyelination and neurodegeneration [55].

Loss of function mutations of the heterodimer G-subfamily transporter ABCG5/G8 cause sitosterolaemia [56] that is characterised by decreased biliary excretion of dietary plant sterols (phytosterols) resulting in early onset atherosclerosis and heart diseases [57].

Although the majority of ABC proteins transport hydrophobic or amphiphilic compounds, there are also ABC family members that handle hydrophilic substrates. For instance, one isoform of ABCC6 mediates the release of nucleoside triphosphates, predominantly ATP, into the circulation [58]. In the blood ATP is rapidly converted to AMP and inorganic pyrophosphate (PPi) by the ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1). The main action of PPi is to directly inhibit hydroxyapatite formation and thereby PPi is an important mineralization inhibitor [59]. Pseudoxanthoma elasticum (PXE) is a metabolic disorder believed to be caused by the inactivating mutations of ABCC6 [60]. PXE is characterized by a generalized late-onset ectopic calcification of several tissues including eyes, blood vessels and the skin [61].

Interestingly, the homodimer G-subfamily member ABCG2 is an important player of the intestinal uric acid elimination processes [62]. Certain mutations of ABCG2 (e.g., Q141K) decrease the cell surface expression of the transporter [63] resulting in elevated uric acid concentration in the blood (hyperuricemia) and increased risk of gout [36]. Gout is a painful inflammatory arthritis resulting from mono sodium urate crystal deposition in synovial fluid.

Certain ABC transporters are involved in the transport of various bile components including bile salts, phosphatidyl choline (PC), cholesterol and metabolic side products across the canalicular membrane of hepatocytes [64,65]. For instance, ABCB11 is a bile salt export pump (also called BSEP), which exports bile salts into bile. Loss of function mutations of ABCB11 cause the accumulation of toxic bile salts inside the hepatocytes and is responsible for the disease known as familial intrahepatic cholestasis type II [66,67].

ABCC7 is a channel protein conducting Cl⁻ ions across epithelial cell membranes [68–70]. The decreased function or reduced expression level of ABCC7 (CFTR) protein may lead to imbalance of the epithelial fluid transport in many organs including the lungs, the pancreas and the intestinal epithelium and results in a multi-organ disease called cystic fibrosis (CF) [71]. The primary cause of death in CF patients is a progressive lung disease caused by the

formation of a mucus with abnormal composition creating favourable conditions for serious respiratory infections due the decreased mucociliary clearance activities of airway epithelial cells [72]. In 85% of CF cases, deletion of one phenylalanine amino acid at the position of 508 induces misfolding of the protein and its subsequent degradation in proteasomes resulting in the lack of functional CFTR chloride channels in the apical membrane of epithelial cells [73].

ABCC8 and ABCC9 (sulfonylurea receptor 1 and 2 (SUR1 and SUR2), are ion-channel regulators that form complexes with inward rectifying potassium channel (Kir6) subunits to build functional ATP-sensitive K⁺ channels (K_{ATP} channels) [74,75]. ABCC8 is primarily reported in pancreatic β -cells, where it is involved in the regulation of insulin secretion. Loss-of-function mutations of the channel complex are related to congenital hyperinsulinism, while gain-of-function mutations lead to neonatal diabetes [76,77]. ABCC9 mainly occurs in cardiovascular system and smooth muscle cells, and its certain mutations seem to be implicated in cardiomyopathy and Cantú syndrome, while certain gene variants might be associated with age-related neurodegenerative diseases [78].

Interestingly, the majority of the above-discussed ABC protein related diseases (and those summarized in **Table 1**) are brought about by mutations that cause protein misfolding, which subsequently may induce degradation of ABC proteins in proteasomes, reducing their cell surface expression [79]. Recent research demonstrated that small molecule chemical chaperons may correct protein folding and trafficking defects [80]. Development of chemical repair for folding mutant variants of CFTR and other ABC proteins demonstrated that chemical chaperons (e.g., 4-PBA) might be applied broadly to improve the function/expression of mutant ABC proteins [81]. These studies opened new avenues for development of chemical chaperons to restore protein function and expression [81,82].

Table 1. Examples of human diseases associated with mutations of ABC proteins

Transporters	Function	Disease	Ref
ABCA1	cholesterol & phospholipid transport	Tangier disease & familial HDL deficiency	[51,83,84]
ABCA2	drug resistance	abnormal sphingolipid metabolism, linked to Alzheimer's disease	[85,86]
ABCA4	photopigment transport in cones and rods	Stargardt disease and age-related macular degeneration	[87,88]
ABCB1	drug resistance	polymorphism is associated with leukaemia and Crohn's disease	[89–91]
ABCB2	peptide transport	immune deficiency	[92,93]

ABCB3	peptide transport	immune deficiency	[92,93]
ABCB4	bile acid transport	progressive familial intrahepatic cholestasis type-3	[94,95]
ABCB6	iron transport	unknown	
ABCB7	iron transport	sideroblastic anaemia and ataxia	[96]
ABCB11	bile acid transport	progressive familial intrahepatic cholestasis type-2	[97]
ABCB12	ceramide transport	Harlequin ichthyosis	[53]
ABCC1	drug resistance	unknown	[98]
ABCC2	bile acid transport	Dubin–Johnson syndrome	[99]
ABCC4	nucleoside transport	unknown	
ABCC6	ATP transport	Pseudoxanthoma elasticum	[61,100]
ABCC7	chloride ion channel	cystic fibrosis	[101,102]
ABCC8	sulfonylurea receptor-1, component of ATP-sensitive potassium channel complex in beta cells of pancreas	persistent hypoglycaemia of infancy	[28,103]
ABCC9	sulfonylurea receptor-2, component of ATP-sensitive potassium channel complex	cardiomyopathy dilated type 1O, Cantú syndrome	[28,104,105]
ABCD1	long chain fatty acid transport into peroxisomes	adrenoleukodystrophy	[106]
ABCE1	oligoadenylate binding protein	unknown	[107]
ABCG1	cholesterol transport	unknown	[83]
ABCG2	drug resistance, uric acid transport	predispose to gout disease	[108]
ABCG5	sterol transport	sitosterolaemia	[109,110]
ABCG8	sterol transport	sitosterolaemia	[110]

2.5. Expression profile and physiological function of human ABCB1

ABCB1 can expel various structurally unrelated lipophilic or amphiphilic compounds from cells including exogenous and endogenous toxins [111]. Expression of ABCB1 in the apical plasma membrane of cells has been reported in many tissue barriers [112], such as intestinal epithelium, blood-brain barrier, the biliary membrane of hepatocytes, adrenal glands, kidney proximal tubule cells, Sertoli cells forming the blood-testis barrier and placental trophoblasts (**Figure 1**) [113,114]. A recent study revealed that besides the placental trophoblasts, components of the foetal membrane (i.e. the innermost tissue layers of placenta forming the intrauterine cavity) also express ABCB1 and play a role in the protection of the developing embryo from toxic materials [115]. Collectively, the tissue expression pattern and

the broad substrate spectrum of ABCB1 imply that it is an important contributor in the protection of the organism against toxic xenobiotic compounds by excreting them into urine, bile and the intestinal lumen, and by preventing their accumulation in critical organs such as the brain or testis. In addition, it plays an important role in the absorption, distribution, elimination, and toxicity (ADME-Tox) of chemotherapeutic drugs used for the treatment of various diseases [116,117].

Interestingly, under physiological conditions no major phenotypical effects were observed in *Abcb1a* knock-out mice and Collie dogs lacking functional ABCB1 transporters in the blood-brain barrier [118,119]. However, serious neurotoxic effects were reported when animals were treated with ivermectin and vinblastine (ABCB1 substrates), suggesting that ABCB1 is an important player in the protection of the central nervous system (CNS) from exogenous toxic compounds [114,120,121].

ABCB1 also express in fair amount in stem cell populations such as hematopoietic stem cells and in stem cell-like cancer cells found in different tumour types (also called drug tolerant persister cells) [122,123].

2.6. Structure of human ABCB1

The human ABCB1 consists of 1,280 amino acids in a single polypeptide chain with a molecular weight of 170 kDa. It is a structurally complete ABC transporter consisting of two TMDs (TMD1 and TMD2) and two NBDs (NBD1 and NBD2). Each TMD comprises of 6 transmembrane α helices (TMs) connected to one hydrophilic NBD [21]. The N- and C-terminal halves are connected by a peptide sequence (see **Figure 4A**) often referred as “linker” region (residues 627–688) [124]. The “linker” is very flexible as no structural information has been obtained yet by cryogenic electron microscopy (cryo-EM) or X-ray crystallography. Accordingly, secondary structure prediction algorithms suggest complete absence of stable structural elements in this region. It contains several phosphorylation sites and thus, it is involved in the regulation of the transporter activity [125,126].

Each TMD is directly linked to the respective NBD by an intracellular loop (ICL) as it is shown in **Figure 4A**., and also non-covalently by short ICLs (ICL1-ICL4). ICL1 (connecting TM2 and 3) and ICL4 (connecting TM10 and 11) are in contact with NBD1, while ICL2 (linking TM4 and 5) and ICL3 (linking TM8 and 9) are in contact with NBD2, as shown in **Figure 4A** [127]. The ICLs make connections between the TMDs and the Q- and X-loops situated at the upper surfaces of the NBDs (see **Figure 4B**) [41].

In accordance with “domain swapping”, the TMs form two bundles (wings) containing TMs from both TMDs. One of the wings consists of TMs 1-3,6,10,11, while the other consists of TMs 4,5,7-9,12 (**Figure 4A**), and they collectively form a large internal cavity within the lipid bilayer that is involved in substrate binding and release.

Based on recent structural studies, the accessibility of this substrate binding cavity periodically changes upon the transport cycle of ABCB1. The inward-facing (IF) conformation of ABCB1 form an inverted “V”- shape (**Figure 4C**), with a large cavity in the centre that is accessible from the intracellular space and also from the plasma membrane. This conformation provides access for substrates to enter and interact with the drug-binding cavity. The “outward-facing” (OF) conformation is characterized by the close proximity of the NBDs and the opening of the central cavity to the extracellular space (**Figure 4E**). The proposed role of the OF conformation is the release of substrates to the extracellular space [128,129].

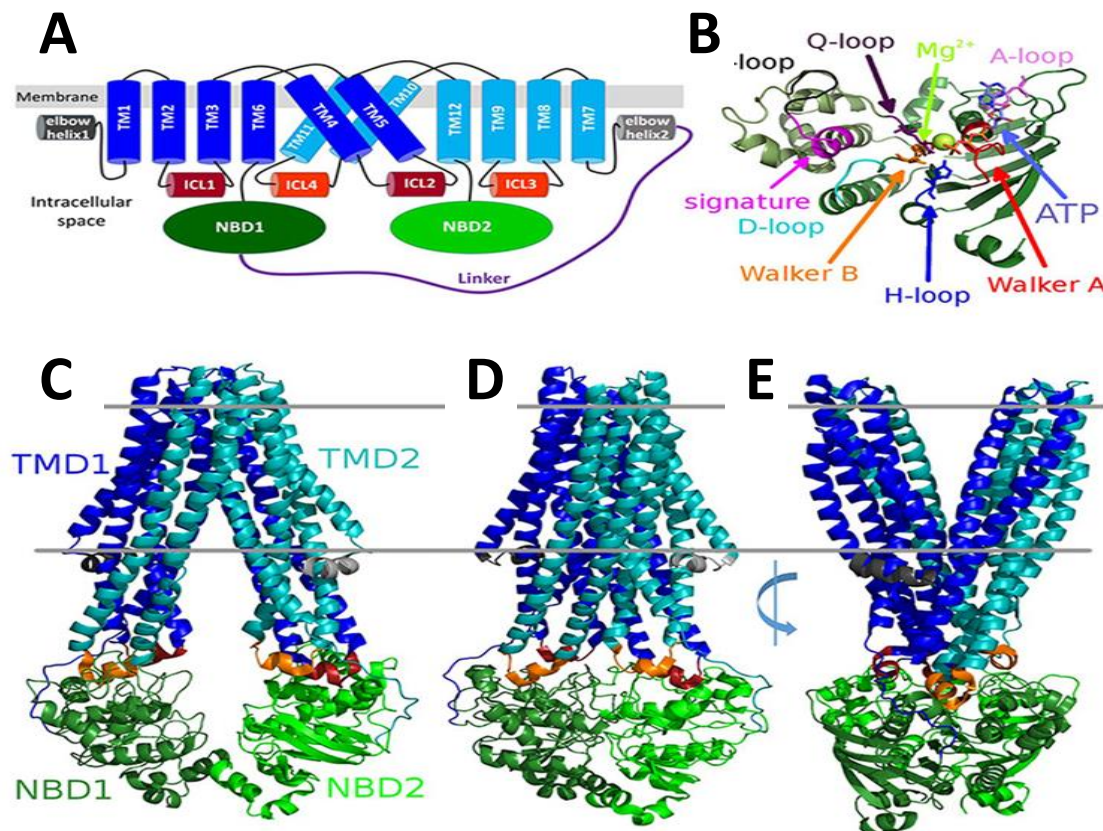


Figure 4. Structural features of ABCB1. Topological model of the ABCB fold (**A**) and focused view of the NBDs highlighting the conserved motifs with bound Mg^{2+} and ATP (**B**). Crystal structures of IF (**C**), occluded (**D**) and OF (**E**) conformations of MsbA, a bacterial homologue of ABCB1 (based on structures PDB ID: 5TV4, 5TTP, 3B60 and [129]). The structures (**C**, **D** and **E**) are coloured according to the topological model (**A**).

In addition to IF and OF conformations, transporter structures in an occluded state are also available. The occluded structures probably represent a transition state in the transport cycle, where both the EC and the IC sides are closed (**Figure 4D**). In the future, cryo-EM studies carried out at turn-over conditions in the presence of substrates and ATP/Mg²⁺ may allow researchers to identify other transient conformations of ABCB1. In general, X-ray or cryo-EM structures serve very important starting points for planning mutagenesis studies and biochemical experiments. In addition, they are also essential for molecular dynamic (MD) simulations as starting structures for simulations to investigate several mechanistic aspects of transporter function. **Table 2** summarizes the most recent structural studies of ABCB1.

Table 2. Summary of ABCB1 structures obtained by X-ray crystallography and cryo-EM based on <https://www.rcsb.org/>.

Source	Ligand	Method (resolution)	Orientation	PDB ID	Remark	Release date and ref
Human	taxol, zosuquidar	cryo-EM 3.40Å- 3.9 Å	Four conformational states	9CR8, 9CTC, 9CTF, 9CTG	Distinct states trapped in absence of antibodies & mutations	2025-01-22,[130]
Cyanidioschyzon merolae strain 10D	Decyl-beta-D-malto-pyranoside, 2-amino-2-hydroxymethyl-propane-1,3-diol	X-RAY diff. 3 Å	Outward-facing	7VR5	Assessing substrate binding by using Trp fluorescence	2022-09-07, [131]
Mouse/Human	UIC2-Fab, encequidar	cryo-EM 3.50 Å	Inward-facing	7O9W	Encequidar is a dual ABCB1 and CYP3A4 inhibitor	2022-01-12, [132]
Mouse	ivacaftor	cryo-EM 5.40 Å	Inward-facing	7OTG	The affinity of ivacaftor to human and murine ABCB1 is similar	2021-12-08, [133]
Mouse/Human	tariquidar, cholesterol, vincristine, elacridar, zosuquidar	cryo-EM 3.2Å- 3.9 Å	Outward-facing or inward facing depending on ligand binding	7A69, 7A65, 7A69, 7A6C, 7A6E, 7A6F	Vincristine (antitumor drug) is a substrate of ABCB1, while the potent inhibitors elacridar, tariquidar, or zosuquidar. can act as substrates at low concentration and at higher concentration	2020-10-21, [134]

					bind at two sites leading to inhibition.	
Mouse	polybrominated diphenyl ether)-100 (4C8)	X-RAY diff. 3.98–4.17 Å resolution range	inward facing	6UJN, 6UJP, 6UJR, 6UJS, 6UJT, 6UJW	Compensatory recognition forms of polyspecific drug transport determine by aromatic amino acids.	2020-05-27, [135]
Human/Mouse	TAXOL, Zosuquidar, Cholesterol, 2-acetamido-2-deoxy-beta-D-glucopyranose	cryo-EM 3.6 Å	Inward-facing	6QEX, 6QEE, 6QEX		2019-02-27, [136]
Cyanidioschyzon merolae strain 10D	Decyl-beta-D-malto-pyranoside	X-RAY diff. 1.9 Å–3.02 Å	Inward- and outward-facing	6A6M, 6A6N	An outward-facing conformational state with bound nucleotide and an inward-facing apo state	2019-02-20, [137]
Mouse	ADP	cryo-EM 7.9 Å	Inward facing	6Q81, 6GDI	Post-hydrolytic state, ADP remains bound	2018-12-26, [138]
Human	ATP/Mg ²⁺	cryo-EM 3.4	Outward-facing	6C0V	The two nucleotide-binding domains form a closed dimer occluding two ATP molecules.	2018-01-31, [139]

2.7. Transmembrane domains and their role in substrate binding

It is believed that ABCB1 evolved from an ancient half-transporter containing only one NBD and TMD by a gene duplication event followed by randomly occurring point mutations resulting in a pseudo-symmetric protein [140]. In a study comparing the amino acid sequence of TMDs and NBDs in mouse ABCB1, it was observed that the evolution of the two TMDs was more divergent compared to the NBDs. There is 77.3% sequence similarity between the two NBDs, while the TMDs diverged more exhibiting only 43.5% sequence similarity [141]. The TMs collectively form a large substrate-binding cavity with an approximate volume of 6,000 Å³ that spans almost the entire width of the lipid membrane (**Figure 5**) [124]. The amino acid residues interacting with substrates come from TM1, TM4, TM5, and TM6 of TMD1 and TM7, TM10, TM11 and TM12 of TMD2. The inner surface of the drug binding pocket is rich in aromatic and hydrophobic residues and also include a few polar and charged amino acid side chains.

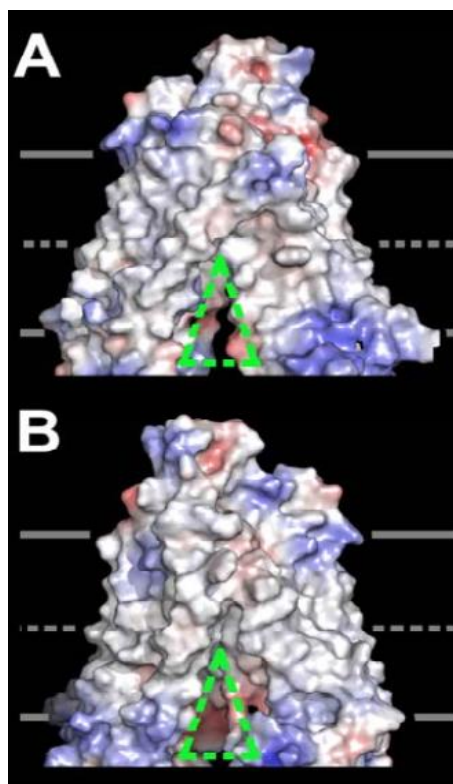


Figure 5. Substrate-entry portals of mouse ABCB1. (A) Stereo-view of ABCB1 within the lipid bilayer. (B) View rotated 180° from (A) showing the portal on the opposite side of ABCB1. Solid lines delineate the approximate location of lipid headgroups, while dashed line represents the ends of the lipid acyl chains located halfway in the bilayer. The surface of ABCB1 is coloured according to variation from blue (positively charged regions) to white (hydrophobic and aromatic) to red (negatively charged regions). A green triangle highlights the opening of the substrate-entry portal [124].

The exact mechanism by which ABCB1 recognizes numerous structurally different substrates is not completely identified, yet. Using [125I]-iodoaryl azidoprazosin, a photoactive analogue of the ABCB1 substrate prazosin, two regions of the transporter were labelled supporting the presence of two non-identical drug-interacting sites within the substrate binding cavity [142-144]. In another study applying substrate transport assays Shapiro A. B. and Ling V. proposed that ABCB1 may possess three drug binding sites (**Figure 5**): the H site is selective for Hoechst 33342 and colchicine, the R site binds rhodamine 123 and anthracyclines and a third binding site recognizes progesterone and prazosin (M site), which has a positive allosteric effect on substrate transport by the H and R sites [145,146]. Recent molecular docking experiments demonstrated that each site (H, R and M) can bind verapamil, doxorubicin or colchicine [147], as it shown in (**Figure 6**).

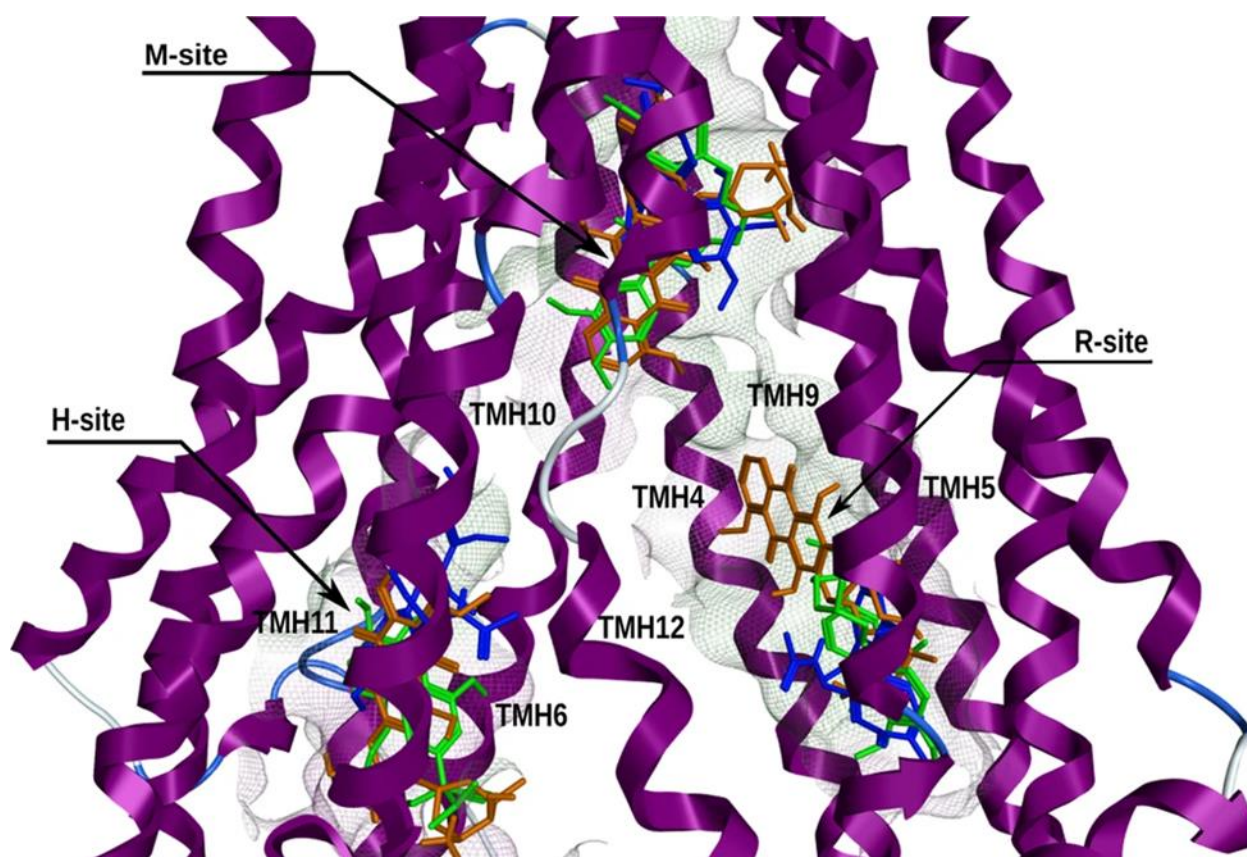


Figure 6. Representation of the binding sites for verapamil (green), doxorubicin (dark orange) and colchicine (blue) found within the large drug binding pocket/cavity of human wild-type ABCB1. The three drug binding sites are defined by the best-ranked docking poses at each binding site, based on [148].

Besides the application of fluorescent substrates and photo-affinity labelling experiments, radioligand-binding assays were also exploited to characterize the drug interaction sites of ABCB1. Based on the observation of competitive (common site) or non-competitive (distinct sites) interactions between ligands a minimum of four drug binding sites were proposed [149].

Loo and Clark exploited cysteine mutagenesis experiments combined with thiol-reactive ABCB1 substrates to identify the amino acid residues involved in substrate binding. These studies demonstrated that the binding sites of several drugs including cyclosporine A (CsA), tariquidar or valinomycin spatially overlap with each other. Their experimental findings were explained by formulation of the “substrate-induced fit” model stating that the complex substrate-binding pocket can accommodate structurally different substrate molecules by exposing different amino acid residues on the surface of the substrate-binding pocket as a result of substrate-specific rotational and lateral movements of the involved TM segments [150,151].

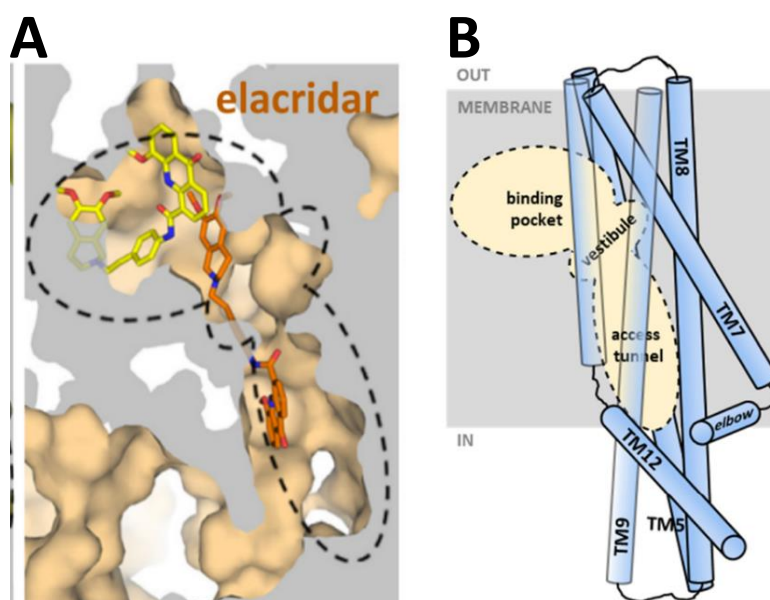


Figure 7. Elacridar binding to human ABCB1 (**A**) and schematic representation of the central drug-binding pocket, vestibule, and access tunnel within ABCB1 (**B**). The transmembrane helices (TM) that surround the access tunnel are shown as blue cylinders. The pale-yellow area surrounded by dashed line correspond to the area shown in panel A., based on [134].

Collectively, the above-described findings strongly supported the existence of multiple substrate-binding sites in the substrate-binding pocket of ABCB1. However, these studies could not determine the exact number of simultaneously bound substrates and could not specify the spatial localization of the substrate binding sites. More recently, X-ray crystallography and cryo-EM studies proved that ligands bind to partially overlapping sites inside the large central binding cavity [152–154]. Moreover, structures of different inhibitor-ABCB1 complexes suggest, that ABCB1 inhibitor molecules often bind in pairs. For instance, the elacridar- (**Figure 7A**) and zosuquidar (ZQU)-bound cryo-EM structures of ABCB1 showed that one inhibitor molecule binds to the central drug-binding pocket, while a second molecule extends to a phenylalanine-rich cavity termed "access tunnel", that is located between the central cavity and the cytoplasmic gate region formed by the kinks in TM4 and TM10 (**Figure 7B**). In general, inhibitor molecules fill up the substrate-binding cavity and form more interactions with the neighbouring amino acid residues as compared to substrates. These high-affinity ligand-ABCB1 interactions may have long-range effects on the overall conformation of the protein that can hinder or even prevent NBD-dimerization [155] and the subsequent steps of the catalytic cycle resulting in the inhibition of ATPase and transport activity [152].

2.8. Transport models explaining the working mechanism of membrane transporters

In general, three models were formulated for explaining the working mechanism of membrane transporters. The so-called “pump model” assumes that substrates interact with the membrane exporter in the cytoplasmic aqueous phase, and then they are moved across the lipid bilayer, and are finally released into the extracellular aqueous phase [156]. This model can offer perfect explanation for the transport of hydrophilic compounds that cannot partition into the lipid membrane significantly [156].

However, substrates of ABCB1 (and many other ABC proteins) are amphiphilic or lipophilic compounds (such as verapamil) characterized by high $\log P_{ow}$ values (i.e. octanol-water partition coefficient) and thus they avidly partition to the lipid bilayer and often reach several thousand-fold higher concentrations in the membrane compared to the aqueous phase.

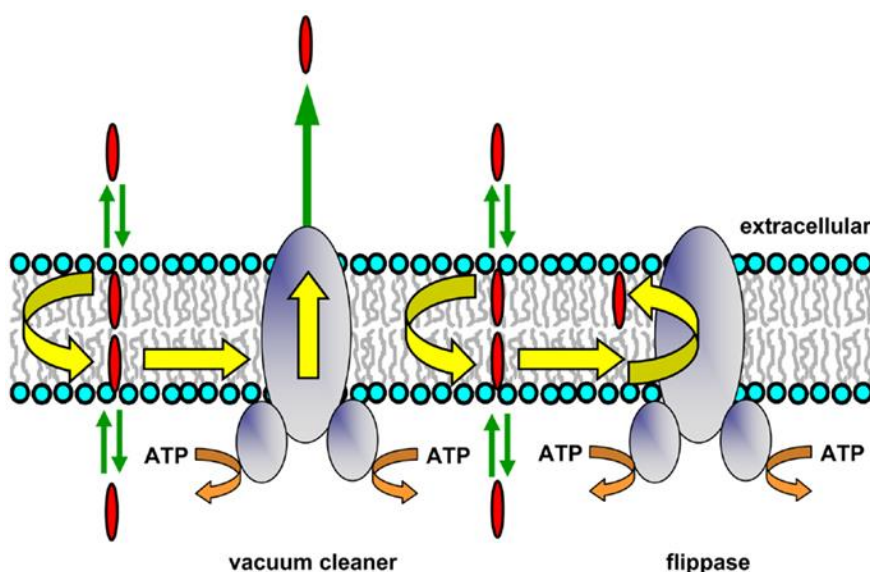


Figure 8. “Hydrophobic vacuum cleaner” and “flippase”/“floppase” models of ABCB1 function. In both models, substrates partition into the membrane, spontaneously translocate to the inner leaflet, and gain access to the substrate-binding pocket of ABCB1 from within the bilayer interior. In the “hydrophobic vacuum cleaner” model, substrates are directly extruded into the extracellular aqueous phase, while in the “flippase”/“floppase” model they are translocated to the outer membrane leaflet [157].

It was also found that the apparent K_m values of the ABCB1-mediated transport of compounds strongly correlate with their hydrophobicity [157]. Based on these observations, Gottesman and colleagues postulated the “hydrophobic vacuum cleaner model” [156]. In this model, hydrophobic drugs first partition into the inner leaflet of the plasma membrane from where ABCB1 takes them and transports to the aqueous extracellular milieu (**Figure 8**). This model was supported by transport experiments with fluorescent drugs [158] and more recently by EPR studies employing spin-labelled verapamil [159].

The third model was originally called “flippase” model, however, recently it was renamed to “floppase” model, since according to the latest nomenclature movement of molecules from the inner to the outer leaflet was named as flopping, while the inverse movement was defined as flipping. It suggests that similar to the “hydrophobic vacuum cleaner” model substrates first partition into the inner membrane leaflet from where the transporter captures and “flops” them back to the outer leaflet. From the outer leaflet substrates either diffuse into the extracellular aqueous phase, or flip back to the cytoplasmic leaflet, where they can be recaptured by ABCB1 (**Figure 8**).

Either the “hydrophobic vacuum cleaner” or the “floppase” model was essentially based on experiments showing that drug binding to ABCB1 occurs in the cytoplasmic membrane leaflet [160]. For instance, Omote and Al-Shawi [161] applied an interesting experimental setup. They investigated the transport activity of ABCB1 in proteo-liposomes using a permanently charged spin-labelled verapamil derivative that cannot passively diffuse across the membrane. Labelled verapamil was added to the outside of the vesicles, then partitioned into the outer leaflet of the lipid bilayer, where it was captured by ABCB1 and was “transported” to the inner leaflet with a turn-over number of 5.8/s. This process led to about 10-fold accumulation of labelled verapamil in the inner membrane leaflet of the vesicle and resulted in the development of a high positive surface potential due to the permanent charge of verapamil. It should be noted that the orientation of ABCB1 in vesicles was opposite to the physiological resulting in transport of verapamil from outer to the inner leaflet, whereas in cells transport works from the inner to outer leaflet.

Taken together, it is likely that ABCB1 transports its substrates from the inner membrane leaflet to the outer leaflet or to the extracellular milieu depending on the chemical nature and $\log P_{ow}$ value of substrates.

2.9. Membrane physiochemical properties modulate the function of ABCB1

As an integral membrane protein, ABCB1 is embedded in the plasma membrane, which is a compositionally and biophysically complex lipid-protein matrix. The lipid components of the plasma membrane are extremely diverse, because of their versatile polar head groups, acyl chain lengths, and degrees of saturation [162].

In general, conformation and function of membrane proteins is strongly dependent on their lipid environment. The physicochemical properties of the membrane, such as lateral membrane pressure [163], membrane thickness [164,165] and fluidity [166] are important for the structural stabilization and functionality of membrane proteins, especially membrane

transporters that may undergo complex conformational changes upon their duty cycles. Experiments showed that ABCB1 activity can be modulated by changes in membrane thickness [167], membrane fluidity, membrane melting temperature [168] and membrane composition [169].

In a cryo-EM study aiming to address the direct interactions between membrane components and ABCB1, a “lipid belt” tightly bound to the TMHs of ABCB1 was identified. At the level of the outer membrane leaflet this “belt” consists of ordered cholesterol molecules, while at the level of the inner membrane leaflet, it contains tightly bound phosphatidylethanolamines and cholesterol, as it is shown in **Figure 9**. Removal of these strongly associated annular lipid molecules by detergents upon protein purification led to the inactivation of ABCB1 and several other ABC transporters [170–172].

On the other hand, transport activity of ABCB1 and many other transporters are determined by the availability of their cargos. As it was recognized early on, compounds interacting with ABCB1 are typically lipophilic or amphipathic and accumulate in the membrane at high concentrations. It should be noted that due to this strong membrane sequestration, the measured affinities of ABCB1 for substrate binding from the membrane are in the millimolar range. This low affinity binding to ABCB1 is an important factor in the development of poly-specific substrate recognition. *In silico* molecular docking studies serve good tools for analysing ligand-transporter interactions. Unfortunately, low affinity binding of ligands (especially substrates) to ABCB1 is a burden for molecular docking studies, because scoring functions used in docking are optimized for high affinity binders and largely rely on the enthalpic contribution of ligand binding (personal communication, Thomas Stockner, Medical University of Vienna).

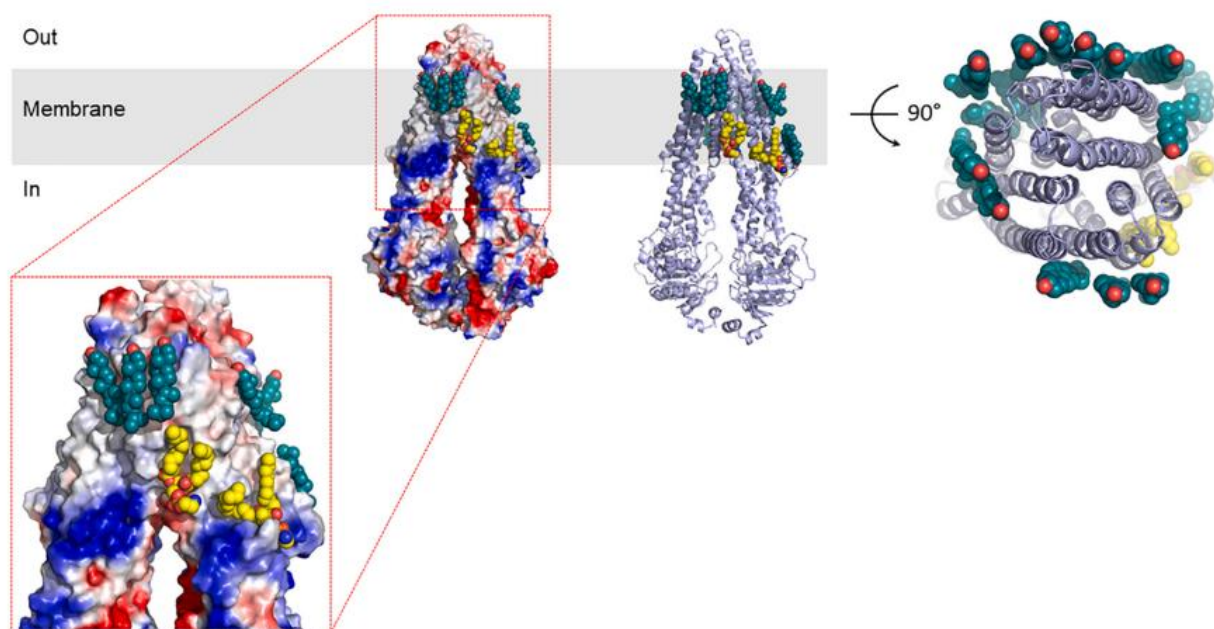


Figure 9. Phospholipids and cholesterol bound to ABCB1. The structures of the transporter are shown either as electrostatic surface potentials (ESPs) or as ribbon diagrams. ESPs are coloured for negative partial charges red and blue for positive partial charges. Tightly bound cholesterol molecules and phosphatidylethanolamine are shown in sphere representation. Cholesterol is coloured green, whereas phosphatidylethanolamine is coloured yellow. The structure was taken from [152].

According to the widely accepted hydrophobic “vacuum cleaner” and “flippase”/“floppase” models, the long residence-time of substrates in the inner leaflet of the plasma membrane facilitates their binding to ABCB1. The ability of ABCB1 substrates to diffuse through the membrane is dependent on the lipid composition and the lateral packing density of the membrane [173]. For instance, it was experimentally demonstrated that the transmembrane flip-flop of an ABCB1 substrate doxorubicin was inhibited by increasing the cholesterol content of the membrane and sped up by high temperatures and the membrane fluidizer benzyl alcohol [174].

2.10. Interactions of polyphenols with ABCB1

Polyphenols are secondary metabolites produced by plants. Over 9,000 polyphenols were identified from different plants [175,176]. They are often involved in the protection of plants from herbivores and pathogens or alternatively they attract pollinators and seed carriers. Polyphenols can be classified into different groups based on their chemical structure, such as phenolic acids, flavonoids, stilbenes and lignans [177–179] (see **Figure 10**). Phenolic acids possess very strong antioxidant properties [180]. Phenolic acids can be divided into two subgroups: (1) hydroxybenzoic acids, such as ellagic acid and (2) hydroxycinnamic acids,

including caffeic acid, chlorogenic acid and ferulic acid among others. Flavonoids are the most abundant polyphenols in the human diet. They contain a chromone ring (benzo-dihydropyran, $C_9H_{10}O$) with an additional aromatic ring attached at position 2, 3 or 4. Based on the different substituents and oxidation state of ring C, flavonoids can be classified into several subclasses, such as flavans (e.g., flavan-3-ols like catechin and epicatechin), flavones (e.g., apigenin, tangeretin, luteolin), flavonols (e.g., quercetin, quercetin-3-glucoside, narcissoside), flavanones (e.g., naringenin), chalcones (e.g., chalconaringenin) and anthocyanidins (e.g., cyanidin, malvidin). Anthocyanins also belong to flavonoids and they are derived from anthocyanidins by adding sugars, such as cyanidin-3-O-sophroside (or cyanidin-3,3'-diglucoside), keracyanin (or cyanidin-3-O-rutinoside), kuromanin (or cyanidin-3-O-glucoside), oenin (or malvidin-3-glucoside) and malvin (or malvidin-3,5-diglucoside).

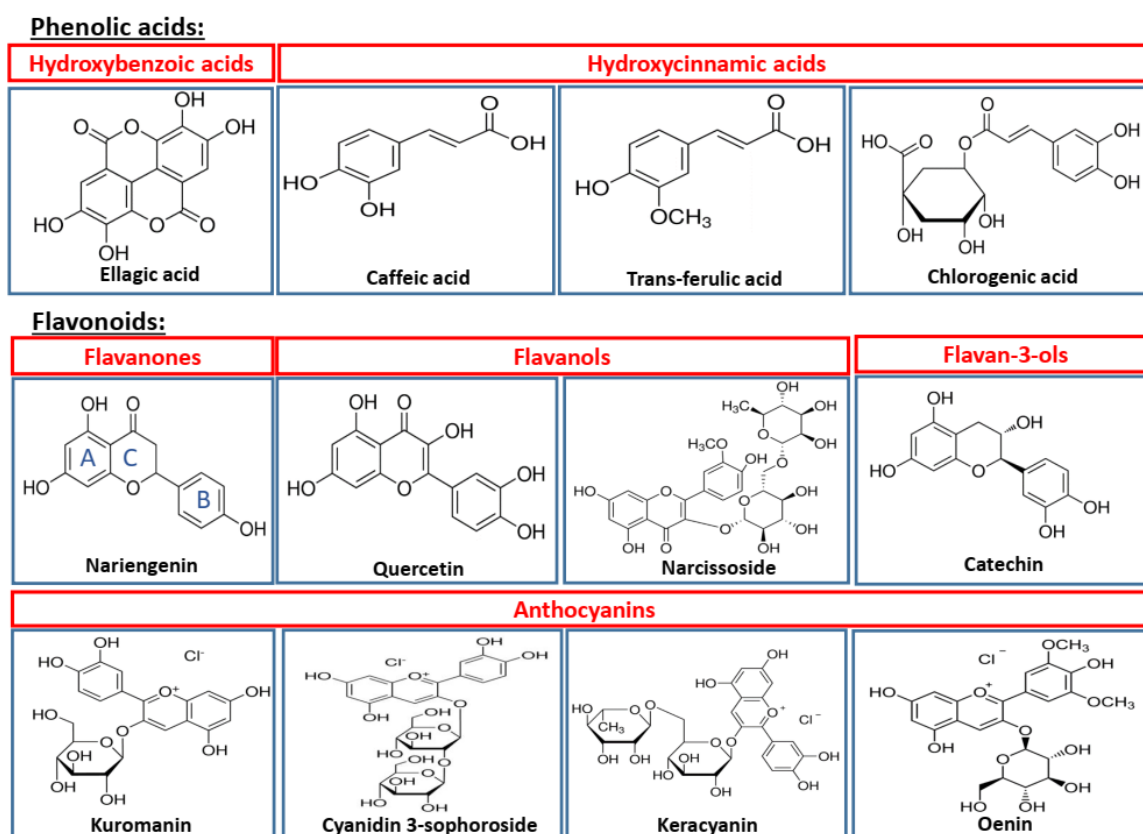


Figure 10. Structures of polyphenols studied in our project. In the structure of the flavanone naringenin the chromone ring (A and C) and the additional aromatic ring (ring B) are also indicated.

Polyphenols are abundant in vegetables, fruits, flowers, coffee and tea. Polyphenol-containing foods and beverages have long been used for their various health benefits mainly related to their anti-oxidant, anti-viral or anti-inflammatory effects [181,182]. Previous research also demonstrated the importance of certain polyphenols in the prevention of

carcinogenesis, cardiovascular problems, neurodegenerative diseases and immune disorders. While foods containing flavonoids are generally considered safe, there have been reports indicating potential food-drug interactions, when polyphenol containing food and certain medications were co-administered [183,184]. For instance, grapefruit juice is known to interact with certain drugs, including calcium channel blockers and midazolam, and can alter both the pharmacokinetic and pharmacodynamic parameters of these drugs [185]. A potential mechanism of food-drug interactions might be that certain polyphenols affect the function of drug metabolizing enzymes (such as cytochrome P450), membrane transporters and receptors, and thereby alter the pharmacokinetics and pharmacodynamics of other co-administered drugs [186]. These interactions can lead to changes in drug efficacy, bioavailability and safety profiles. Therefore, potential food-drug interactions should be considered, especially for drugs with narrow therapeutic indices, like certain calcium channel blockers [187,188]. Since ABCB1 is an important determinant of the pharmacokinetics of numerous chemotherapeutic compounds, studying the interaction of ABCB1 with flavonoids may shed light on potential drug-flavonoid interactions on the level of ABCB1.

Several tested polyphenols such as nobiletin, auraptene, sesamin, ginkgolic acid, matairesinol glycyrrhetic acid, glabridin, and phyllostulcin were found to increase the accumulation of the anticancer drug daunorubicin in ABCB1 expressing KB-C2 cancer cells [189]. In spite of their transport inhibitory effect auraptene and nobiletin increased, while glycyrrhetic acid inhibited the verapamil-stimulated ATPase activity of ABCB1 [190,191]. Other polyphenols, such as ellagic acid have also been reported to have inhibitory effects on ABCB1 [192].

Several papers have reported the ability of certain polyphenols to increase the efficacy of anticancer drugs in multidrug resistant cancer cells by modulating one or more of the three main efflux pumps (ABCB1, ABCG2 and ABCC1) involved in MDR. Interestingly, some flavonoids, like karanjin can act as a broad-spectrum competitive ABC transporter inhibitor interfering with drug efflux mediated by ABCB1, ABCC1 and ABCG2. Furthermore, karanjin was found to be more effective compared to other flavonoids such as apigenin, genistein, and naringenin, on all the three MDR transporters [193].

Amudkar *et. al.* extensively studied the effect of licochalcone A, an extract from the root of Licorice species, on ABCG2 overexpressing multidrug resistant cancer cells and found it both to inhibit drug transport activity and to stimulate ATPase activity [194]. Moreover, *in silico* docking analysis confirmed that licochalcone A binds to the substrate-binding pocket of

ABCG2 [194]. Flavones, such as chrysin and retusin also inhibit the transport activity of ABCG2 [195,196].

Taken together, the large pool of plant-derived polyphenols can be deemed as a “natural source molecule library” that can be used to hunt for potential ABC transporter inhibitors. On the other hand, exploring the interaction of polyphenols with ABC transporters may help to avoid potential “food-drug” interactions on the level of transporters.

3. Aims of the project

Previous studies have demonstrated that certain polyphenols interact with human ABCB1. However, unfortunately the data obtained in different experimental systems are often controversial. Since polyphenols are present in fruits and vegetables at high concentrations and often used in food supplements or as components of cosmetics, their possible interaction with ABCB1 may affect the pharmacokinetics of other co-administered ABCB1 substrate chemotherapeutic drugs. To fully characterize the interaction of 15 dietary polyphenols abundant in red fruits with ABCB1 we set the following aims:

1. Studying the interaction of 15 dietary polyphenols with the human ABCB1 in ATPase activity assays, transport assays and in a UIC2-reactivity test.
2. Measuring the effects of polyphenols on the membrane fluidity in DPH and TMA-DPH anisotropy measurements.
3. Studying the effects of different polyphenol-verapamil combinations on ABCB1 activity.
4. Detailed analysis of the combined interaction of two polyphenols quercetin and cyanidin-3O-sophoroside with ABCB1 by using transport and ATPase assays and *in silico* ligand docking experiments.

4. Materials and Methods

4.1. Chemicals

We purchased all reagents, cell culture media, and supplements used for our research from Sigma-Aldrich (Budapest, Hungary). Fluorescent dyes, including calcein acetoxymethyl-ester (calcein-AM) and Alexa 647 succinimidyl ester (A647), were obtained from Life Technologies, Inc. (Carlsbad, CA, USA). The ABCB1-specific monoclonal antibodies (mAbs) UIC2 and 15D3 were purified from hybridoma supernatants by affinity chromatography. The hybridoma cell lines producing the mAbs were obtained from the American Type Culture Collection (Manassas, VA, USA). The mAbs were labeled with A647, and they were purified from the unconjugated dye by gel filtration using a Sephadex G-50 column. The dye-to-protein labeling ratio was determined for every Ab preparation and was found to be around 2-3. Lipophilic transporter substrates/inhibitors (e.g., calcein-AM and cyclosporine A (CsA)) were dissolved in dimethyl sulfoxide (DMSO), while verapamil was dissolved in distilled water. All tested polyphenols (quercetin (QUR), quercetin-3-glucoside, naringenin, ellagic acid, cynidin-3O-sophroside (C3S), oenin, malvin, kuromanin, keracyanin, caffeic acid, chlorogenic acid, trans-ferulic acid, catechin, epicatechin, and narcissoside) were HPLC grade (with 99% purity) and were purchased from Sigma-Aldrich (Budapest, Hungary). Polyphenols were dissolved in DMSO or distilled water according to the manufacturer's instructions. For all experiments, the final DMSO concentration of samples was less than 1% (v/v).

4.2. Cell lines

The NIH 3T3 mouse fibroblast cell line and its human ABCB1 overexpressing counterpart, NIH 3T3 MDR1 [197] were kindly provided by Michael Gottesman (National Institutes of Health, Bethesda, MD, USA). NIH 3T3 and NIH 3T3 MDR1 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 0.1mg/ml penicillin–streptomycin cocktail. Both cells lines were cultured at 37°C in a humidified 5% CO₂ incubator and were passaged every two days. Cells were regularly tested and were found to be negative for mycoplasma infection using the MycoAlert® mycoplasma detection kit (Lonza Rockland Inc., Rockland, ME, USA).

4.3. Harvesting cells from culture flasks

We used trypsin-EDTA (0.25% trypsin-EDTA in PBS) in all experiments to detach cells from the wall of cell culture flasks. First, the culture media was completely removed from the cell culture flask. Cells were rinsed 3 times with 2 mL of ice-cold Phosphate Buffered Saline

(PBS, pH=7.4) to remove cell debris and cell culture media. 2 mL of trypsin-EDTA was then added, and the flask was transferred to a 37°C water bath for 2-3 min. Detachment of cells was facilitated by gently tapping the side of the flask. 4 ml of cold PBS with 10% FBS was added to the detached cells to stop the proteolytic effect of trypsin, and then the cells were transferred into a 15 ml centrifuge tube to centrifuge them at $435 \times g$ for 5 minutes. After centrifugation the supernatant was discarded, and the cells were washed one more time by PBS and then resuspended in cold PBS containing 7 mM glucose (gl-PBS).

4.4. Substrate accumulation tests

To study the effect of all 15 polyphenols on the transport activity of ABCB1, we applied the fluorescent ABCB1 substrates calcein-AM and daunorubicin. The intracellular accumulation of calcein and daunorubicin increases in response to partial or complete inhibition of ABCB1 [198,199]. Samples containing 0.5×10^6 cells/mL were pre-treated with 10 μ M CsA, 40 μ M verapamil, and/or the tested polyphenols (10-150 μ M) for 10 min at 37 °C and then further incubated with 3.8 μ M daunorubicin for 30 min or 0.5 μ M calcein-AM for 20 min. After the incubation, samples were washed three times with ice-cold gl-PBS containing 0.5% fetal bovine serum (FBS) and were kept on ice until flow cytometry measurement.

4.5. UIC2 and 15D3 reactivity measurements

The ABCB1 overexpressing NIH 3T3 MDR1 cells (5×10^5 cells/ml) were pre-treated with 10 μ M CsA, 40 μ M verapamil or different polyphenols in gl-PBS for 10 min at 37°C, and then further incubated with 10 μ g/mL UIC2-A647 mAb at 37 °C. After 30 min of incubation, samples were washed 2 times with ice-cold gl-PBS and centrifuged for 5 min at $435 \times g$ at 4 °C. The UIC2-A647 fluorescence intensity of the cells was measured by flow cytometry. To determine the ABCB1 expression of cells, we used the conformation-insensitive 15D3-A647 mAb (30 μ g/ml) and followed the same procedure for Ab labelling as for UIC2.

4.6. Membrane preparations from NIH 3T3 cells

For ATPase activity measurements, we used crude membrane samples derived from ABCB1 overexpressing and ABCB1 negative NIH 3T3 cells prepared according to Sarkadi's method with slight modifications [200]. To avoid degradation of proteins, all steps of the membrane preparation procedure were carried out at 4 °C in the presence of protease inhibitors including phenylmethylsulphonyl fluoride (PMSF), and protease inhibitor cocktail (PIC, Sigma-Aldrich, Budapest, Hungary). Cell homogenization was performed by a glass-Teflon tissue homogenizer in TMEP buffer solution (50 mM Tris-HCl (pH = 7.0), 50 mM mannitol,

2 mM EGTA, 0.5 mM PMSF and PIC (applied according to the instruction of the manufacturer). After cell homogenization, intact cells and cell nuclei were sedimented at $500 \times g$ for 10 min at 4 °C and discarded, while the resulting supernatant was kept and further centrifuged at $28,000 \times g$ for 60 min at 4 °C to pellet membrane fragments. The resulting pellet containing membrane fragments was resuspended in TMEP solution and stored at -70 °C until use. The protein concentration of the membrane samples was determined by the Lowry method [201].

4.7. ATPase activity measurements

The ABCB1-specific ATPase activity of the membrane samples was calculated by measuring the amount of inorganic phosphate (Pi) released upon ATP hydrolysis. Other ATPase proteins, which are abundant in cells, were blocked by inhibitors, such as Na-azide for the F0F1 ATPases, ouabain for Na⁺/K⁺-ATPase, and EGTA for the Ca²⁺-ATPases [35,200]. Membrane samples (5 µg membrane protein/sample) were pre-incubated with different concentrations of polyphenols with or without 40 µM verapamil in 60 µL ATPase assay premix (50 mM MOPS, 65 mM KCl, 6.5 mM Na-azide, 2.6 mM DTT, 1.28 mM ouabain, 0.65 mM EGTA, pH = 7.0) in the presence or absence of 100 µM Na₃VO₄ (vanadate) for 5 min at 37 °C. Thereafter the ATPase reaction was initiated by the addition of 3.2 mM MgATP. After 25 min incubation at 37 °C, the ATPase reaction was stopped by 40 µL 5% SDS, and then the samples were incubated with 105 µL color reagent at room temperature (22°C) for 30 min. The color reagent was prepared from 5 ml of Pi-reagent (containing 1% ammonium molybdate, 0.014% potassium antimony tartrate, and 2.5M H₂SO₄), 3 ml of 20% acetic acid and 2.5 ml of 1% ascorbic acid [202]. The absorbances were measured at 700 nm using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT, USA), and the amount of Pi was calculated for all samples. Finally, the ABCB1-specific ATPase activity was determined by calculating the differences between vanadate-positive and vanadate-negative sample pairs.

4.8. Measurement of membrane fluidity

The effects of polyphenols on the membrane fluidity were studied in ABCB1 overexpressing and non-expressing NIH 3T3 cells. Samples containing (1×10^6 cells/ml in gl-PBS) were pre-treated with polyphenols applied at 100 µM concentration for 10 min at 37 °C. After 10 minutes the membrane specific probes 2 µM diphenylhexatriene (DPH) or 2 µM 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) were added to the samples and further incubated at room temperature for 20 min in dark. Steady-state

fluorescence anisotropy measurements were carried out at 37 °C using a Horiba Jobin Yvon Fluorolog-3 (Yvon Horiba, Edison, NJ, USA) spectrofluorometer equipped with a thermostated cell holder. For the anisotropy measurements the fluorescence of DPH and TMA-DPH was excited at 358 nm and their emission was measured at 427 nm. The steady-state fluorescence anisotropy of the dyes was calculated using the formula:

$$r = \frac{I_{VV} + GI_{VH}}{I_{VV} - 2GI_{VH}}$$

wherein I_{VV} and I_{VH} are the vertically and horizontally polarized components of the fluorescence intensities, respectively, excited by vertically polarized light and G is a correction factor compensating for the unequal sensitivity of the detection system for vertically and horizontally polarized light. There is an inverse correlation between membrane fluidity and fluorescence anisotropy. Lower anisotropy value indicates higher membrane fluidity, while higher fluorescence anisotropy shows lower fluidity and higher structural order within the membrane [203].

4.9. Flow cytometry

Calcein and daunorubicin accumulation measurements were carried out using the Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Calcein was excited by the 488 nm line of a solid-state laser, and the emitted light was detected using a 502 nm dichroic mirror and a 530/30 nm band-pass filter. Propidium iodide (PI) and daunorubicin were excited at 488 nm, and their fluorescence signal was collected through a 585/42 nm band-pass filter. The fluorescence signal of A647 was detected using a 635 nm red diode laser and a 661/15 nm band-pass emission filter. Dead cells were identified by PI staining and were excluded from the analysis. All fluorescence signals were collected in logarithmic mode, and the cytofluorimetric data were analyzed using the Flowing software (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

4.10. Statistical analysis

We used SigmaPlot (version 14.0, SPSS Inc., Chicago, IL, USA) for the statistical analysis of experimental data. Data from $n \geq 3$ independent measurements are presented as means $\pm$ SD. The n values of the different experiments are shown in the figure legends. Comparison of two groups was performed by unpaired t-test, while in the case of three or more groups statistical significance was assessed using analysis of variance (ANOVA). For *post hoc* pair-wise comparison of the treatment groups with identical variances the Holm-Sidak multiple

comparison test was applied, while groups with unequal variances were analyzed by Dunnett T3 post hoc test. Differences were considered significant at $P < 0.05$.

4.11. Molecular docking

The cryo-electron microscopy-based (cryo-EM-based) structure of human ABCB1 (PDB ID: 7A6F, Resolution: 3.50 Å) in complex with the ligand zosuquidar (ZQU) was used for molecular docking studies. The amino acid residues not visualized in the cryo-EM structure (1-31, 86-103, 631-693 & 1277-1280) were modelled using Modeller 9.25 [204]. During the preparation of the protein structure for *in silico* docking studies, the bound ZQU and the water molecules were eliminated. Subsequently, hydrogen atoms were introduced, and their positions were optimized using the Tinker 8 program, with a gradient norm set to 0.05. [205] The correct protonation states to each residue were assigned using the PROPKA program [206,207] The 2D structures of the co-crystallized ZQU) and the other studied ligands cyanidin-3-sophroside (C3S) and quercetin (QUR) were drawn and subsequently transformed to 3D structures in Marvin Sketch 5.6.0.0 program (<http://www.chemaxon.com>) program. The resulting 3D structures were geometry optimized after assigning Gasteiger charges in UCSF Chimera 1.8 [208] program with the combination of steepest descent and conjugate gradient geometry algorithms with 100 steps (step size 0.05 Å), and a conjugate gradient method with 10 steps (step size 0.01 Å).

Molecular docking studies were performed with AutoDockVina [209]. Grid boxes large enough to encompass each co-crystallized pose of ZQU were set up. For the central drug binding site (site 1) the grid box dimensions were 14, 12, and 16 Å. For the access tunnel (site 2), the grid box dimensions were 9, 15, and 12 Å. The ABCB1 structure was rigid during docking. In order to validate the docking protocol, two ZQU molecules were docked sequentially at the two sites. The docked ZQU molecules almost completely reproduced the conformation of the co-crystallized ZQU-ABCB1 complex. The RMSD values were well below 2 Å indicating that the docking protocol including the setup of grid box dimensions and grid centre are appropriate for further docking studies.

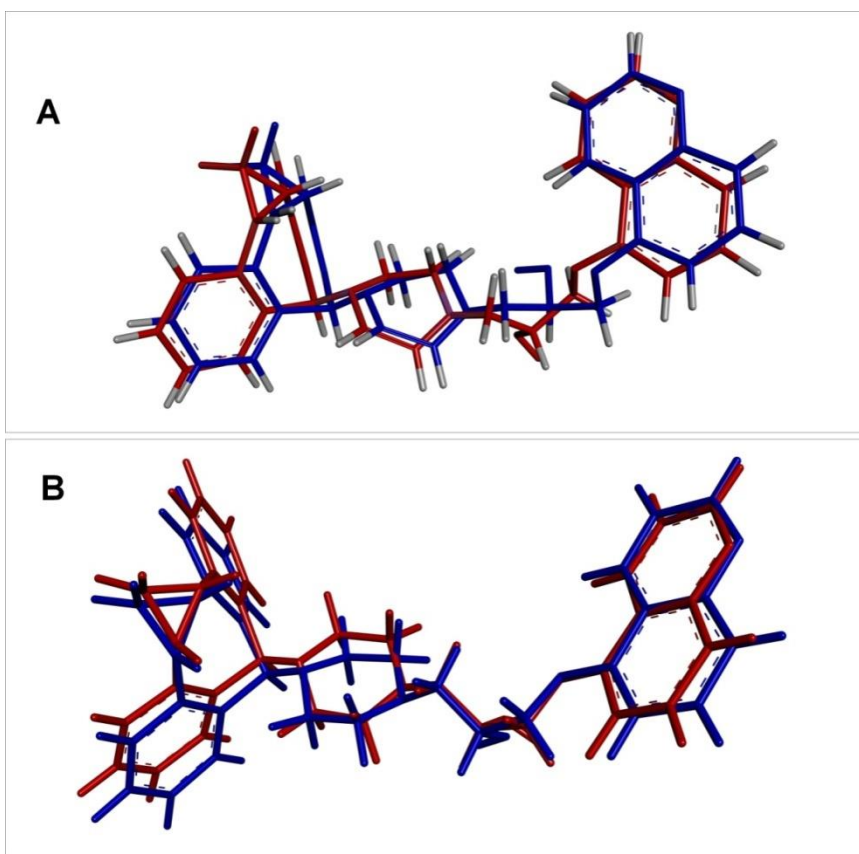


Figure 11. Docked (blue) and cryo-EM (red) poses of ZQU at site 1 (**A**) and at site 2 (**B**) in the drug binding cavity of ABCB1.

Similarly, C3S and QUR were docked at each site with the same parameters, which were used for ZQU. For instance, one of these two ligands was docked at site 1 and docking scores were evaluated. In the next step, keeping the best pose of this ligand at site 1, the other ligand (C3S or QUR) was docked at site 2. In order to investigate the entire space of the binding cavity the exhaustiveness was set to 100. The ligand poses were ranked based on their docking score.

5. Results

5.1. Section I

5.1.1. Effects of polyphenols on the ATPase activity of ABCB1

NIH 3T3 MDR1 cells express ABCB1 at high level in their plasma membrane, as we demonstrated by flow-cytometric immunofluorescence assay using 15D3-A647 mAb (**Figure 12**), and therefore, this cell line is convenient for identifying potential ABCB1 substrates or inhibitors. We have analysed the effects of 15 dietary polyphenols on the ATPase activity of ABCB1 using membrane samples prepared from NIH 3T3 MDR1 cells (**Figures 13-15**). We studied the effects of polyphenols on the basal and substrate stimulated ATPase activity of ABCB1. Basal ATPase activity was measured in the absence of ligands, while substrate stimulated ATPase activity was determined in the presence of verapamil, a known ABCB1 substrate [200]. In control experiments we titrated the concentration of verapamil and observed that 40 μ M verapamil induced the maximum stimulation of the ABCB1-specific ATPase activity (**Figure 13F**). Therefore, verapamil was applied at 40 μ M concentration in all experiments, when the effect polyphenols was tested on the so-called verapamil stimulated ATPase activity. Polyphenols were categorised into three sub-groups based on their behavior in ATPase measurements.

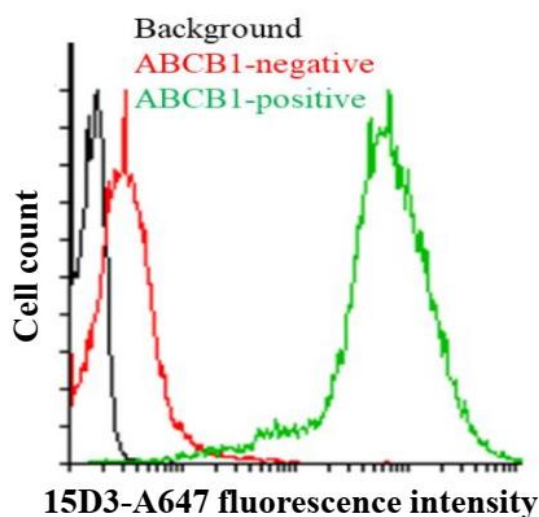


Figure 12. Direct immunofluorescence labeling of ABCB1-positive NIH 3T3 MDR1 cells and the parental, ABCB1-negative NIH-3T3 cells by 15D3-A647 mAb.

Sub-group 1 involving cyanidin-3O-sophoroside (C3S), kuromanin, naringenin, caffeic acid and catechin stimulated the basal ATPase activity of ABCB1, while decreasing the verapamil stimulated ATPase activity (**Figure 13A-E**). Caffeic acid and catechin had weaker effect on the ATPase activity compared to the others (**Figure 13D and E**). It is interesting to

note that all these compounds with similar effect on ABCB1 activity belong to different subgroups of flavonoids (see **Figure 10**). These observations suggest that structurally unrelated compounds may have similar effects on ABCB1 function.

Sub-group 2 including quercetin (QUR), quercetin-3-glucoside, and ellagic acid strongly decreased both the basal and verapamil-stimulated ATPase activity of ABCB1 (**Figure 14 A-C**), while narcissoside had weaker effect (**Figure 14D**), that did not show concentration dependence. According to general experience compounds that slow down the rate of basal ATPase activity and also inhibit substrate stimulation are ABCB1 inhibitors that are not transported by ABCB1 [210,211].

Sub-group 3 contains polyphenols that did not have any effect on the basal ATPase activity including chlorogenic acid, trans-ferulic acid, malvin, and oenin (**Figure 15**). Notably, epicatechin (**Figure 15C**), which is a stereoisomer of catechin (**Figure 13D**), also belongs to this inactive subgroup of polyphenols. Oenin and malvin slightly decreased the verapamil-stimulated ATPase activity of ABCB1 at high concentrations (**Figure 15D and E**), while keracyanin induced a slight increase, when it was applied at 10 to 50 μ M concentrations (**Figure 15F**).

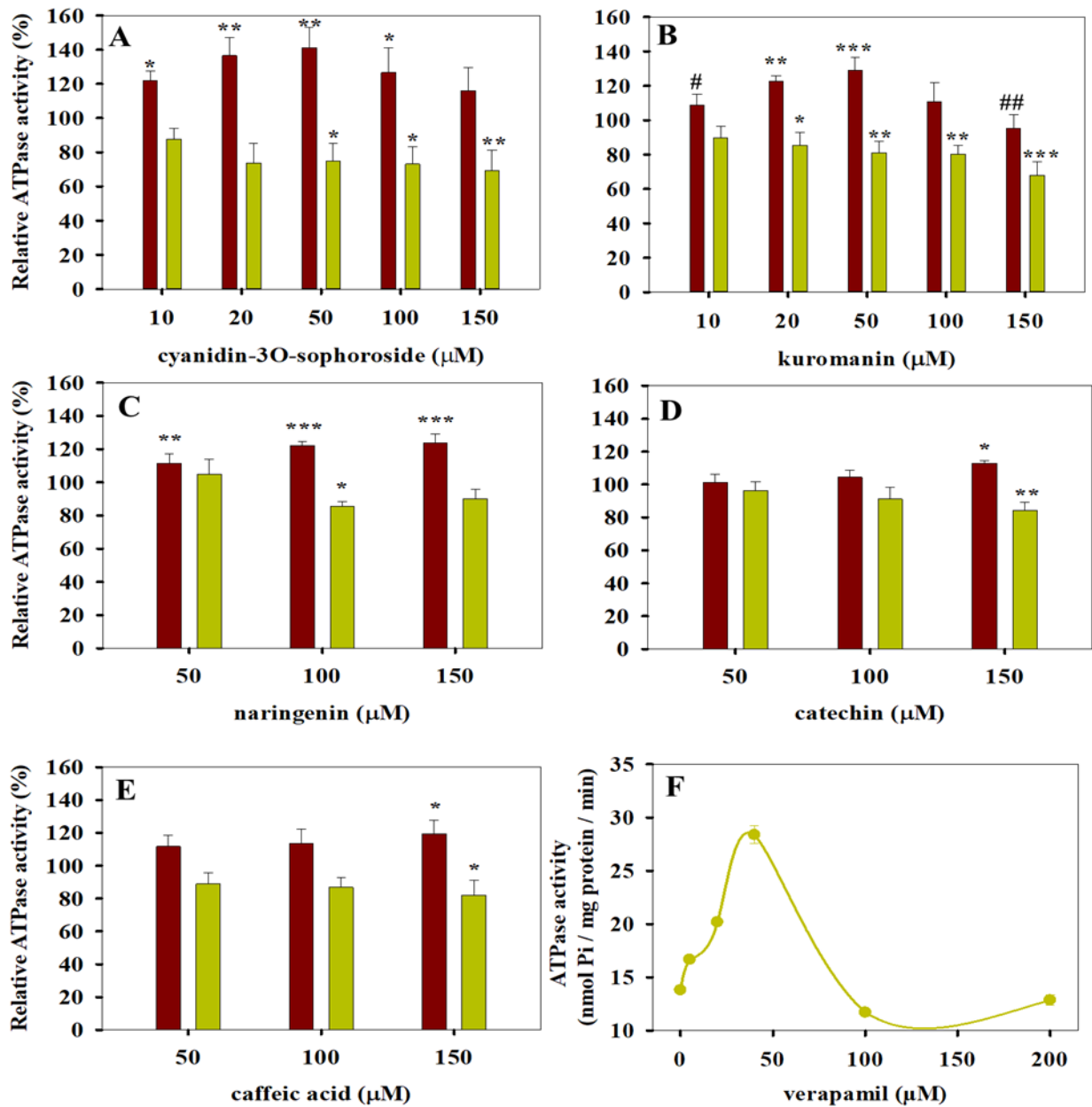


Figure 13. Polyphenols increasing the basal and decreasing the verapamil stimulated ATPase activity of ABCB1 (A–E). The effect of polyphenols on the basal and substrate-stimulated ATPase activities of ABCB1 were measured in the absence (red bars) or in the presence (yellow bars) of 40 μM verapamil, respectively (A–E). Verapamil concentration was titrated in a broad range to find the concentration inducing the maximal stimulation of ABCB1-specific ATPase activity in NIH 3T3 MDR1 membrane samples (F). Relative ABCB1-specific ATPase activities were calculated compared with the polyphenol-untreated samples. Bars represent the means ($\pm$ SD) of 3 independent experiments each performed in triplicates. Significant differences compared with the polyphenol-untreated samples are shown by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Significant differences relative to the 50 μM kuromanin-treated cells are indicated by ##: $p < 0.01$, #: $p < 0.05$.

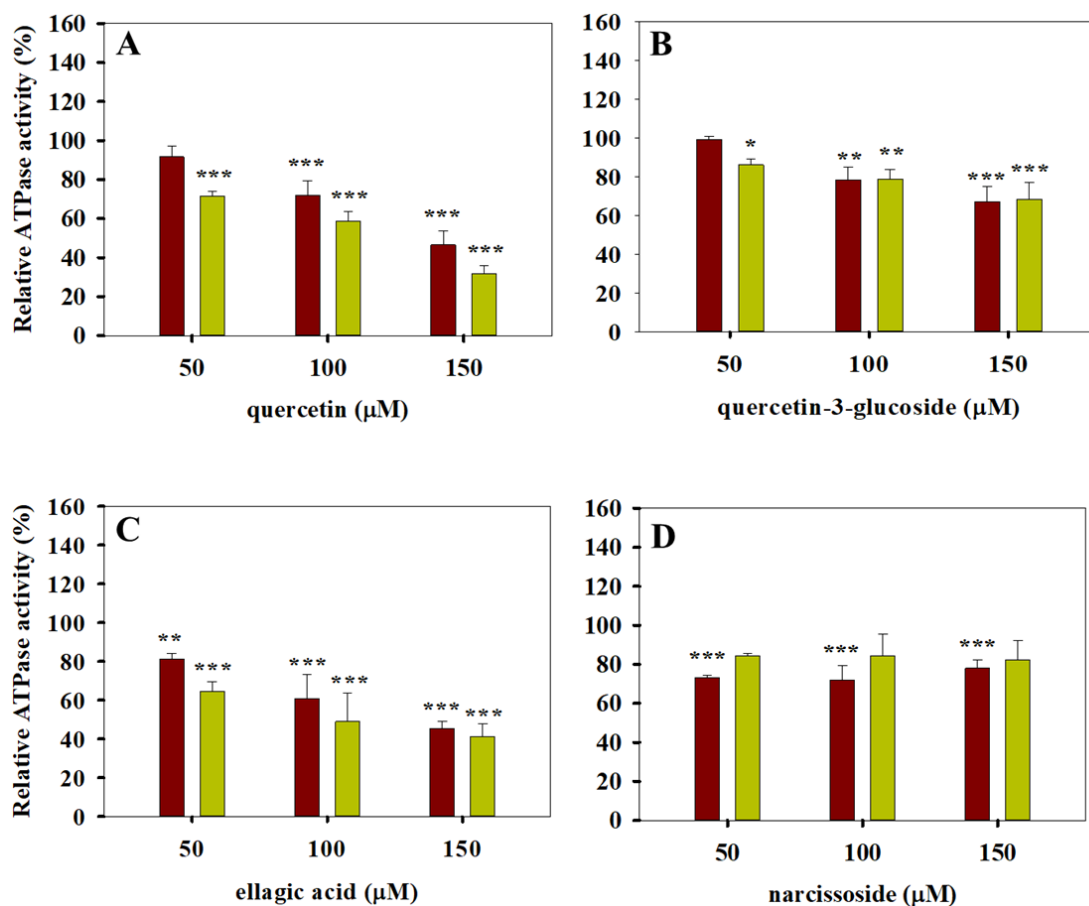


Figure 14. Polyphenols decreasing both the basal and the verapamil stimulated ATPase activity of ABCB1. Basal and substrate-stimulated ATPase activities of ABCB1 were measured in the absence (red bars) or the presence (yellow bars) of 40 μM verapamil, respectively (A–D). Relative ABCB1-specific ATPase activities were calculated compared with the polyphenol-untreated samples. Bars represent the means (±SD) of 3 independent experiments each performed in triplicates. Significant differences compared with polyphenol-untreated samples are shown by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

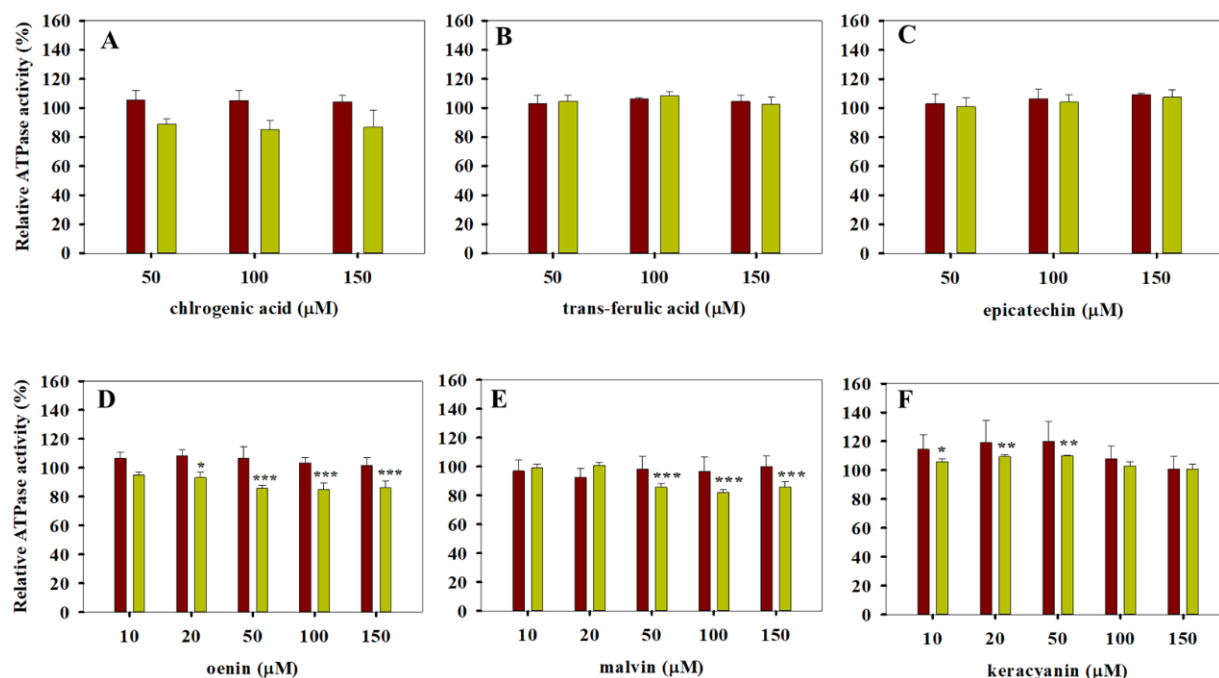


Figure 15. Polyphenols with no significant effect on the basal ATPase activity of ABCB1. Basal and substrate-stimulated ATPase activities were measured in the absence (red bars) or the presence (yellow bars) of 40 μM verapamil, respectively (A–F). Relative ABCB1-specific ATPase activities were calculated compared with the polyphenol-untreated samples. Bars represent the means ($\pm\text{SD}$) of 3 independent experiments each performed in triplicates. Significant differences compared with polyphenol-untreated samples are shown by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

5.1.2. Polyphenols decrease ABCB1-mediated substrate transport

To better understand the interaction of polyphenols with ABCB1, we also performed substrate accumulation experiments with the same ABCB1-positive and negative cell line pair. We applied known substrates, calcein-AM and daunorubicin that are generally used as indicators of ABCB1 activity. Most of the compounds that were found to interact with ABCB1 in ATPase assays (Figures 13-15), also increased the cellular accumulation of the fluorescent calcein and daunorubicin (Figure 16) indicating that they reduce the transport activity of ABCB1.

Among the tested polyphenols, QUR exhibited the strongest inhibition of ABCB1, increasing the intracellular accumulation of calcein and daunorubicin, in a concentration-dependent manner (Figure 16A). In contrast, quercetin-3-glucoside showed weaker effects, consistent with its lower inhibitory effect observed in ATPase assays (compare Figure 16B with Figure 14B). Ellagic acid and naringenin (Figures 16C and D) increased the intracellular accumulation of calcein by 200–250% compared to the control, in agreement with previously

reported data [323, 329]. However, no significant effects of ellagic acid, naringenin, or caffeic acid were observed on the intracellular accumulation of daunorubicin (Figures 16C, D, F).

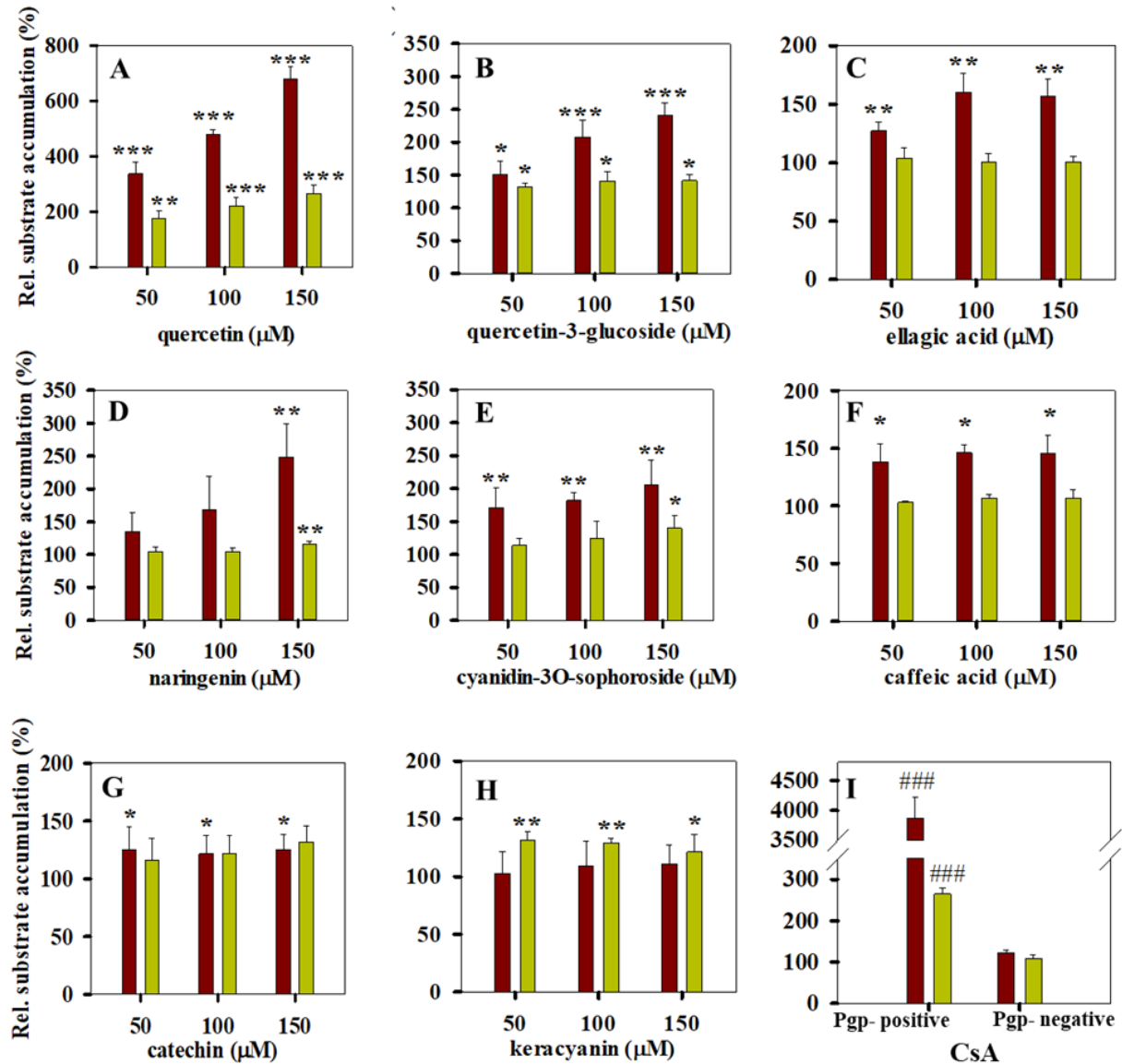


Figure 16. Polyphenols that decrease the transport activity of ABCB1 in calcein (red bars) and daunorubicin (yellow bars) accumulation experiments (panels (A–H)). The effect of 10 μ M cyclosporine A (CsA) on the uptake of calcein and daunorubicin by ABCB1-positive and ABCB1-negative cells (panel (I)). Cells were pre-treated with polyphenols (panels (A–H)) or CsA (panel (I)) for 10 min at 37 $^{\circ}$ C, and then stained with 0.5 μ M calcein-AM or 3.8 μ M daunorubicin for 20 min and 30 min, respectively. The intracellular accumulation of calcein and daunorubicin was measured by flow cytometry. Relative substrate accumulation was calculated by normalizing the mean calcein or daunorubicin fluorescence intensities of the CsA/polyphenol-treated samples to the CsA/polyphenol-untreated control samples and expressed as a percentage (%). Bars represent the means ($\pm$ SD) of 3 to 5 independent experiments. Significant differences compared with the untreated samples are shown by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. In panel I, significant differences compared with the ABCB1-negative cells are shown by ###: $p < 0.001$.

In the same time, keracyanin's behavior was opposite, as it elevated the accumulation of daunorubicin and did not have significant effect on the intracellular accumulation of calcein (**Figure 16H**). Interestingly, C3S, a close relative of keracyanin (belonging to the anthocyanidin family), increased both the calcein and daunorubicin accumulation of ABCB1-positive cells. Catechin had only weak effect on the intracellular accumulation of calcein and daunorubicin (**Figure 16G**) in accordance with its modest effect on the ATPase activity (**Figure 13D**).

The rest of compounds including chlorogenic acid, trans-ferulic acid, malvin, oenin and epicatechin were found to be inactive or had only slight effect in ATPase assays, and they had also negligible effects in substrate accumulation assays (**compare Figure 15 and Figure 17B**). There is one exception, kuromanin affected both the basal and the verapamil-stimulated ATPase activities of ABCB1 (**Figure 13B**), but it did not show significant effects in calcein and daunorubicin assays (**Figure 17B**).

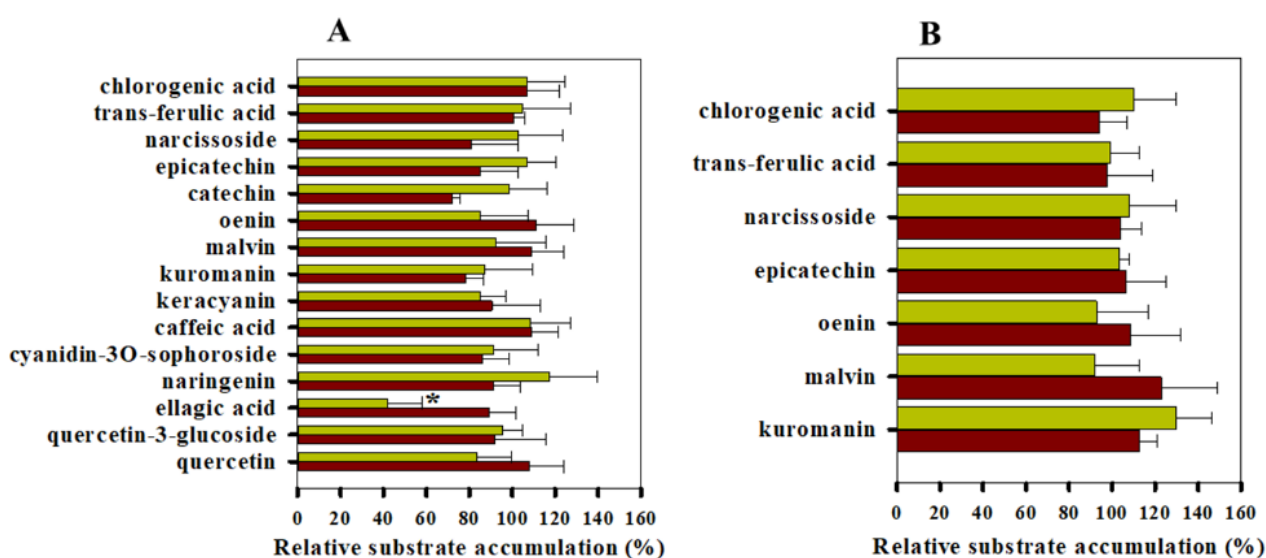


Figure 17. Calcein and daunorubicin accumulation by ABCB1-negative (A) and ABCB1-positive (B) NIH 3T3 cells in response to various polyphenols applied at 100 μ M concentration. Cells were pre-treated with polyphenols for 10 min at 37 $^{\circ}$ C and then further incubated with 0.5 μ M calcein-AM or 3.8 μ M daunorubicin for 20 min and 30 min, respectively. The intracellular accumulation of calcein and daunorubicin was measured in a flow cytometer. The relative substrate accumulation was calculated normalizing the mean calcein or daunorubicin fluorescence intensities of the polyphenol-treated samples to the polyphenol-untreated control samples and expressed as a percentage (%). Bars represent the means ($\pm$ SD) of 3 independent measurements. Significant differences compared with polyphenol-untreated samples are shown by *: $p < 0.05$.

To confirm that the above effects are ABCB1-specific, we also studied the effects of polyphenols on the intracellular accumulation of daunorubicin and calcein in ABCB1-negative cells. Since all studied polyphenols were found to be ineffective in ABCB1-negative control

cells (**Figure 17A**), we concluded that the above-described effects were ABCB1-specific. The only exception was ellagic acid, which strongly decreased the intracellular fluorescence of daunorubicin in ABCB1-negative cells.

5.1.3. Effects of polyphenols on the UIC2 reactivity of ABCB1

Previous studies suggested that efficient ABCB1 inhibitors such as CsA and valsopodar can stabilize ABCB1 molecules in an inward-facing (IF) conformation that is recognized by the conformation selective UIC2 monoclonal antibody [212]. Therefore, to further investigate the interaction of the above polyphenols with ABCB1, we measured UIC2 reactivity with ABCB1 in response to the above-tested compounds (**Figure 18**). Our measurements demonstrated that QUR induced a significant, concentration-dependent increase in UIC2 reactivity (see **Figure 18A**), consistent with its strong ABCB1 inhibitory effect in ATPase and substrate accumulation assays (**Figures 14A** and **16A**). Ellagic acid also increased UIC2 reactivity, albeit no concentration-dependence was detected (**Figure 18A**). It is important to note that other polyphenols exhibiting weaker effects in ATPase and transport assays, did not affect the UIC2 reactivity of ABCB1-positive cells (**Figure 18B**).

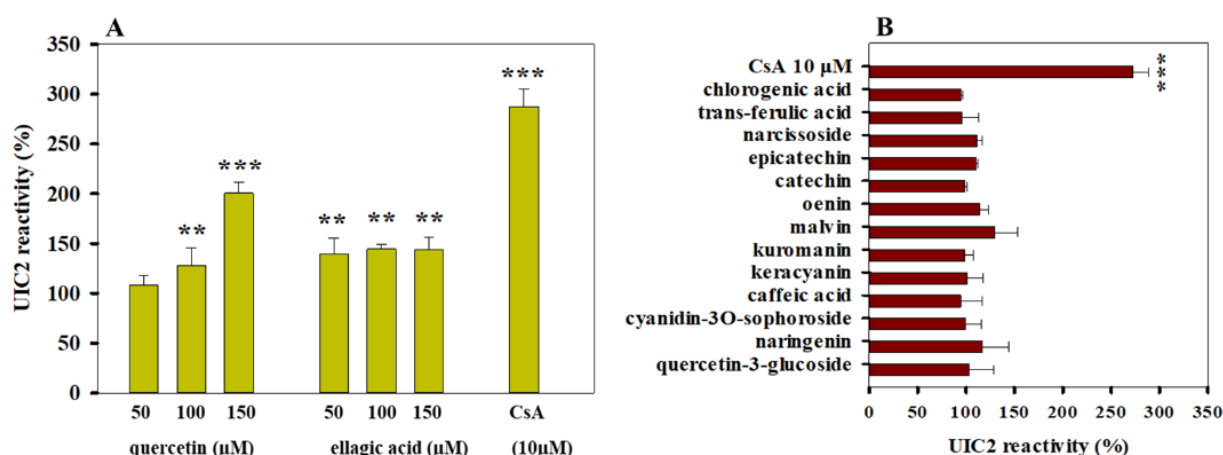


Figure 18. UIC2 reactivity changes in response to polyphenol treatment. Quercetin and ellagic acid were tested in the concentration range of 50–150 μM (**A**), while all other polyphenols were applied at 100 μM concentration (**B**). Cells were pre-incubated in the presence or absence of polyphenols or 10 μM cyclosporine A (CsA) for 10 min at 37 $^{\circ}\text{C}$ and then further incubated with 10 $\mu\text{g/mL}$ UIC2-A647 for another 30 min. The UIC2-A647 staining of polyphenol/CsA-treated cells was normalized to that of the untreated cells. Bars represent the means ($\pm\text{SD}$) of 3 independent experiments. Significant differences compared with untreated samples are shown by ***: $p < 0.001$, **: $p < 0.01$.

5.1.4. Effects of polyphenols on membrane fluidity

It is generally accepted that all transmembrane proteins including ion-channels and transporters are in an intimate relationship with the plasma membrane [213]. There are numerous observations supporting that the lipid composition as well as the physicochemical characteristics of the membrane, such as membrane fluidity can affect the conformation changes and the functional activity of transmembrane proteins. In addition, the substrates of ABCB1 are amphiphilic or lipophilic compounds that can accumulate in the membrane before accessing the substrate binding site of ABCB1. To investigate this issue, we examined the effects of polyphenols on membrane fluidity and packing order. For this purpose we carried out fluorescence anisotropy measurements using the membrane specific probes DPH (6-Phenyl-1,3,5-Hexatriene) and TMA-DPH (1-(4-Trimethylammonium)-6-Phenyl-1,3,5-Hexatriene; **Figure 19**).

DPH is an apolar molecule that accumulates in the inner acyl-chain region of the membrane, while TMA-DPH is a positively charged derivative of DPH [214] that prefer the lipid/water interface of the membrane [214]. Therefore, using TMA-DPH and DPH fluidity and packing order can be estimated in slightly different depths of the plasma membrane, since the average fluorophore of TMA-DPH is about 3–4 Å closer to the membrane surface compared with DPH [215,216]. Fluorescence anisotropy values of DPH and TMA-DPH inversely correlate with membrane fluidity: decreased fluorescence anisotropy indicates increased membrane fluidity, while increased anisotropy corresponds to a decreased membrane fluidity and increased packing order of the membrane. Decreased fluorescence anisotropy values are due to the increased rotational and vibrational freedom of the dye in the plane of the more fluid membrane [215].

The fluorescence anisotropy value of DPH was in the range of 0.18 and 0.20 in untreated ABCB1-positive and negative NIH 3T3 cells (**Figure 19B**), indicating high structural order within the membrane. In contrary, the fluorescence anisotropy of TMA-DPH varied between 0.24 and 0.26, suggesting lower order in the superficial regions of the plasma membrane (**Figure 19A**).

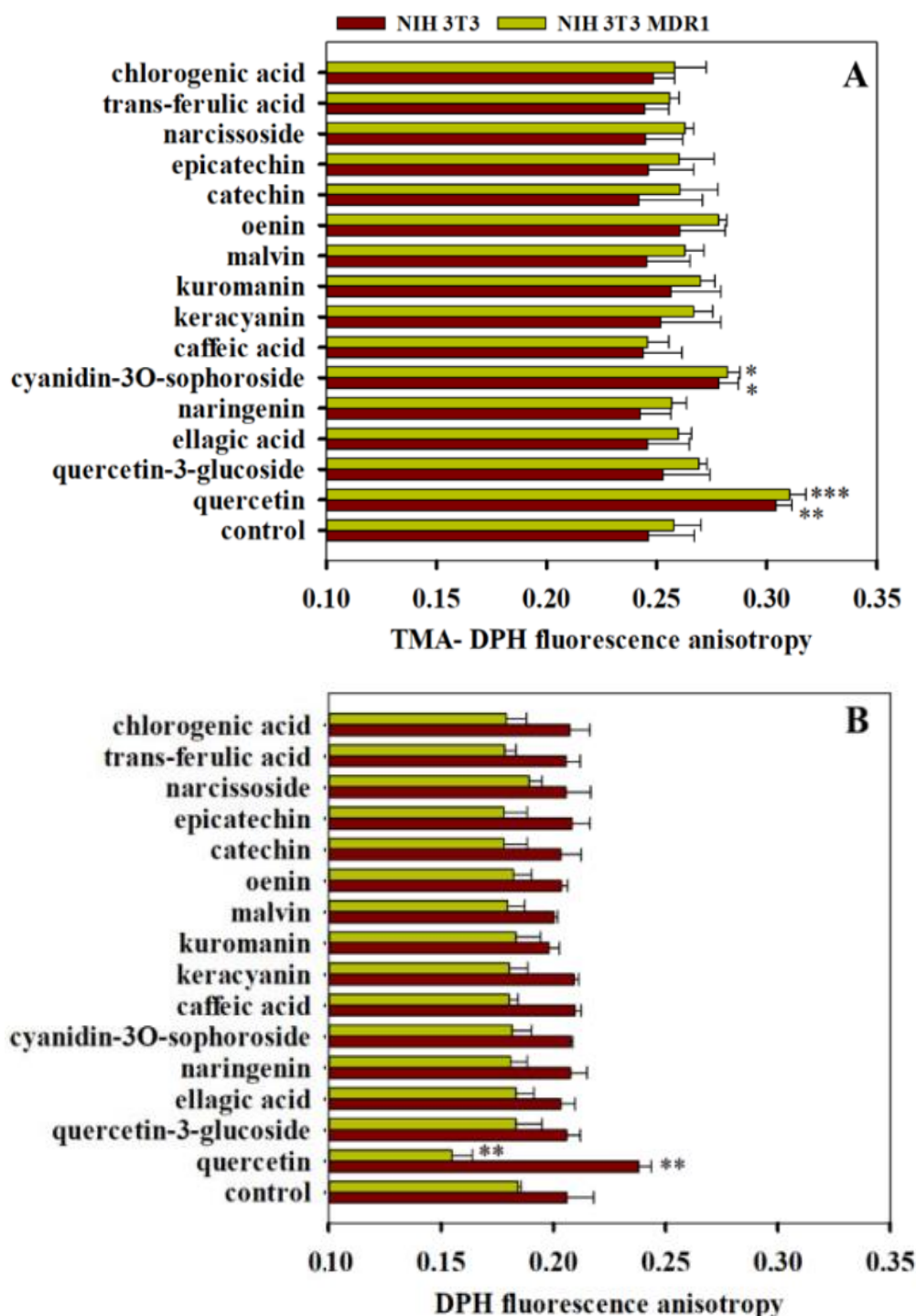


Figure 19. Effects of polyphenols on the steady-state fluorescence anisotropy of TMA-DPH (A) and DPH (B) in ABCB1-positive (yellow bars) and ABCB1-negative (red bars) NIH 3T3 cells. Cells were pre-treated with 50 μ M polyphenol for 10 min at 37 $^{\circ}$ C and then further incubated with 2 μ M DPH or TMA-DPH for another 20 min. Fluorescence anisotropy values were measured at 37 $^{\circ}$ C. Bars represent the means $\pm$ SD of 3 independent experiments performed in triplicates. Significant differences compared with the polyphenol-untreated control samples are shown by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

The measured anisotropy values did not show significant differences between the untreated ABCB1-positive and ABCB1-negative cells, which is consistent with previous

findings [217,218]. QUR and C3S increased the TMA-DPH anisotropy values in both cell lines, indicating a decrease in membrane fluidity at the vicinity of the membrane surface (**Figure 19A**). Notably, in anisotropy measurements carried out with DPH we observed different trends: QUR treatment increased the anisotropy of DPH in ABCB1-negative cells, while a decrease of DPH anisotropy was detected in ABCB1-positive cells. This observation suggests that ABCB1 may have effect on the intramembrane distribution of QUR (**Figure 19B**).

5.1.5. Combined effects of polyphenols and verapamil on the transport activity and UIC2 reactivity of ABCB1

To study the interaction between the known ABCB1 substrate verapamil and certain polyphenols on the level of ABCB1, we performed calcein and daunorubicin accumulation experiments and UIC2 reactivity tests in the simultaneous presence of 40 μ M verapamil and 50-150 μ M concentrations of different polyphenols (**Figure 20**). Combined treatment with QUR and verapamil resulted in higher calcein accumulation compared with the sum of their individual effects, suggesting a possible synergism between them (**Figure 20A**). On the other hand, we observed additive effect of the verapamil-QUR combination in daunorubicin accumulation assay (**Figure 20D**). Verapamil and naringenin showed additive effects both in calcein and daunorubicin accumulation experiments (**Figure 20B, E**). The combination of verapamil and ellagic acid additively increased the intracellular accumulation of calcein (**Figure 20C**). In accordance with the strong ABCB1 inhibitory effect of the combined treatments, we observed elevated UIC2 reactivity compared to the only-verapamil treated samples (**Figure 20 G–I**).

Interestingly, the ellagic acid-verapamil combination decreased the daunorubicin accumulation compared to the only-verapamil treated sample (**Figure 20F**). Since ellagic acid also decreased the daunorubicin staining of ABCB1-negative control cells (see **Figure 17A**), it is likely that ABCB1-independent mechanisms are responsible for this effect. The decreased intracellular daunorubicin staining in response to ellagic acid treatment is probably explained by previous observations that ellagic acid accumulating in the nucleus may form covalent adducts with the DNA [219], and thus it may prevent the binding and sequestration of other DNA-specific drugs, such as daunorubicin.

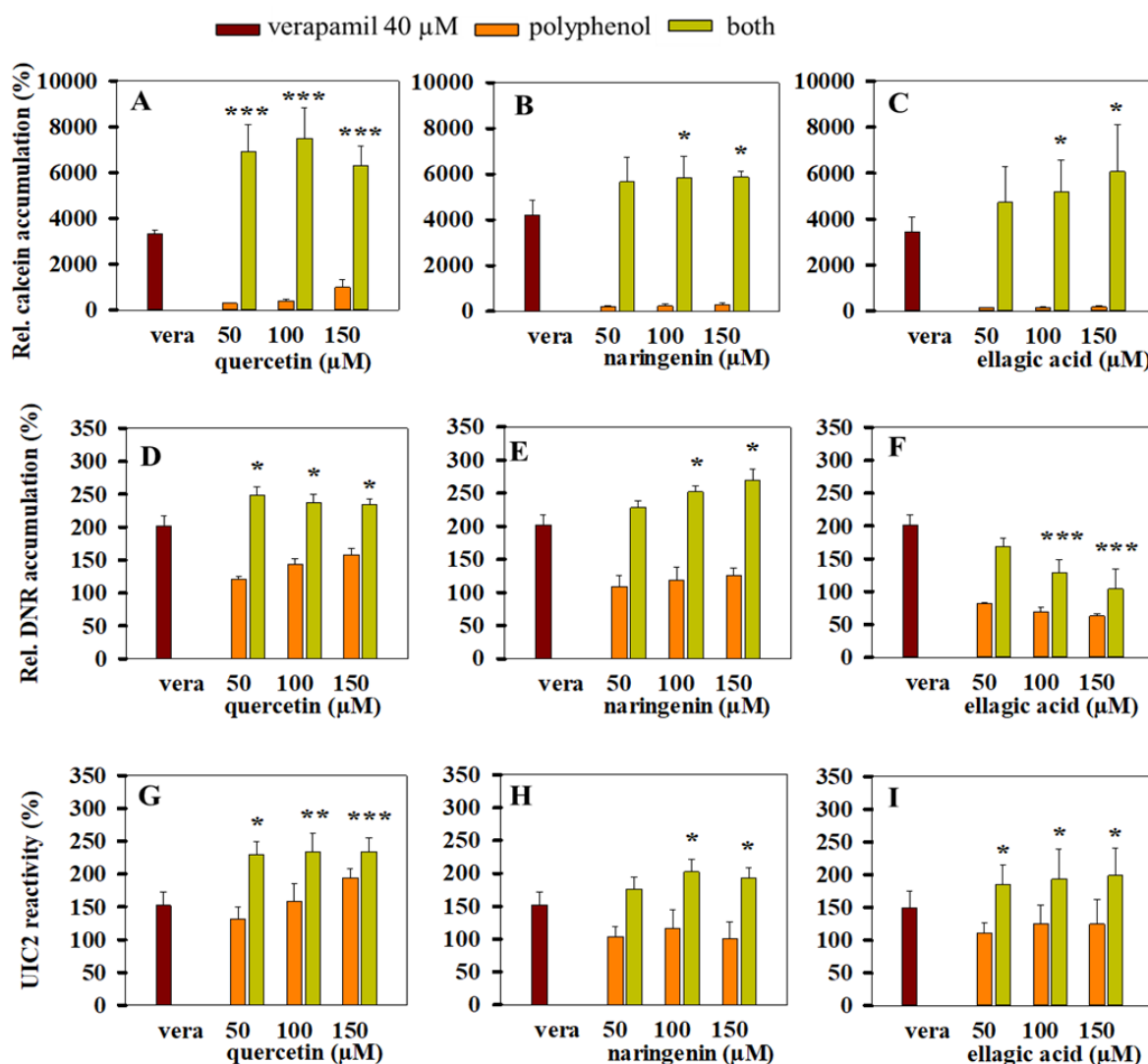


Figure 20. Combined effects of verapamil and certain polyphenols (quercetin (A,D,G), naringenin (B,E,H) and ellagic acid (C,F,I)) on the intracellular accumulation of calcein (A-C) and daunorubicin (D-F) and UIC2 reactivity (G-I) in ABCB1-positive cells. Cells were pre-incubated in the presence or absence of 40 μ M verapamil and/or polyphenols for 10 min at 37 $^{\circ}$ C and then stained with calcein-AM, daunorubicin or UIC2-A647. Bars represent the means ($\pm$ SD) of at least three independent measurements. Significant differences compared with the only-verapamil-treated samples are shown by ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

5.2. Section II.

5.2.1. Combined effect of quercetin and cyanidin-3O-sophoroside on the ATPase and transport activities of ABCB1

In the previous experiments we have found that quercetin (QUR) hampers both the transport and ATPase activity of ABCB1 supporting that it is an ABCB1 inhibitor. On the other hand, cyanidin-3O-sophoroside (C3S) stimulates the ATPase activity and causes only a weak inhibition of substrate transport indicating that it is a substrate of ABCB1 (**Figures 13 and 16**). Since flavonoids and anthocyanins are abundant in fruits and vegetables, we were curious

whether they can reinforce or hinder each other's effect on ABCB1. Interestingly, when QUR and C3S were co-administered, a strong inhibitory effect was observed on ATPase activity, which did not differ significantly from the effect of CsA, a well-known ABCB1 inhibitor (Figure 21).

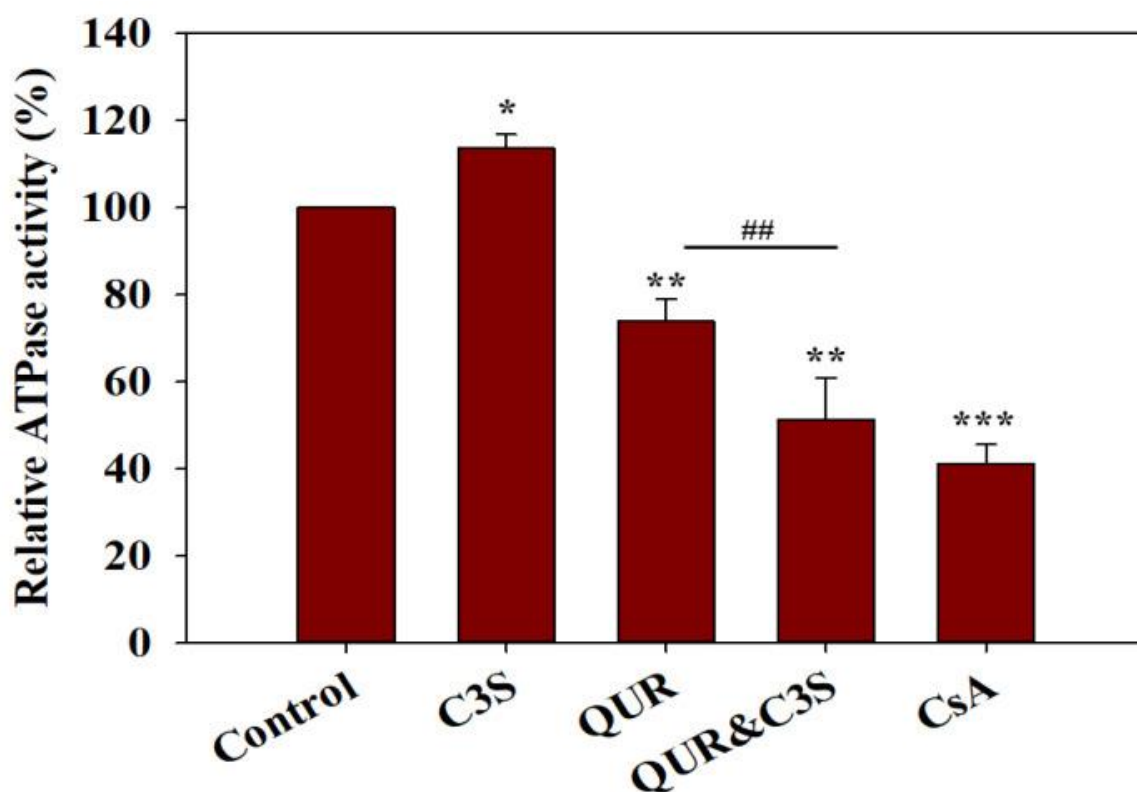


Figure 21. Synergistic inhibitory effects of quercetin (QUR) and cyanidin-3O-sophoroside (C3S) on the ATPase activity of ABCB1. QUR and C3S were applied at 100 μ M, while the known ATPase inhibitor cyclosporine A (CsA) was used at a 10 μ M concentration. Relative ABCB1-specific ATPase activities were calculated relative to the untreated samples. Bars represent the means ($\pm$ SD) of three independent measurements, each performed in triplicates. Significant differences compared to untreated control are shown by ***: $p < 0.001$, **: $p < 0.01$ and *: $p < 0.05$, while significant differences between QUR and the QUR and C3S combination are shown by ##: $p < 0.01$.

To elucidate the effect of QUR, C3S and their combination on the transport activity of ABCB1, calcein and daunorubicin accumulation experiments were carried out. Interestingly, the combination of QUR and C3S, both added at 100 μ M concentration, brought about a strong transport inhibitory effect, which was greater than the sum of the individual effects of each compound (Figure 22A). The observed inhibitory effects were considered ABCB1-specific, since C3S and QUR did not have any significant effect on the calcein and daunorubicin accumulation by the ABCB1-negative NIH 3T3 cells (Figure 22B).

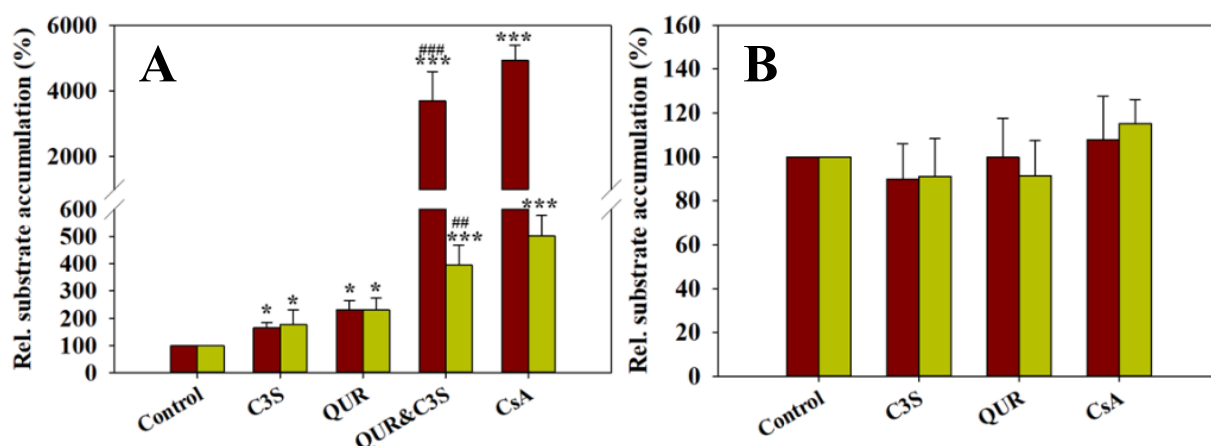


Figure 22. Effects of quercetin (QUR), cyanidin-3O-sophoroside (C3S) and cyclosporine A (CsA) on the calcein (red bars) and daunorubicin (yellow bars) accumulation by ABCB1-positive NIH 3T3 MDR1 cells (A) and ABCB1-negative NIH 3T3 cells (B). Significant differences compared to untreated control are shown by ***: $p < 0.001$, **: $p < 0.01$ and *: $p < 0.05$, while significant differences between QUR and the QUR&C3S combination are indicated by ###: $p < 0.001$ and #: $p < 0.01$.

5.2.2. Effects of QUR and C3S on the conformational state of ABCB1

Complete inhibition of ABCB1 is often accompanied by its stabilization in a UIC2 reactive IF conformation state [220,221]. When added individually, the two polyphenols hardly affected the UIC2 reactivity of the ABCB1-positive NIH 3T3 MDR1 cells (see **Figure 18**). However, an almost three-fold increase of the UIC2 reactivity was detected when the two compounds were applied simultaneously at 100 μ M concentration, in accordance with the strong ABCB1-inhibitory effect of the C3S-QUR combination (**Figure 23**).

The above findings confirm that although QUR alone has only limited ability to stabilize the IF conformer of ABCB1 for recognition by UIC2 mAb, the C3S-QUR combination interacts differently with ABCB1. The observed synergistic effect of C3S and QUR both in functional assays and the conformational assay suggests that these molecules may bind simultaneously to the substrate-binding pocket of ABCB1. To further elucidate the molecular details of the interactions between ABCB1 and its ligands, we conducted *in-silico* molecular docking and molecular dynamics (MD) simulation experiments with QUR and C3S.

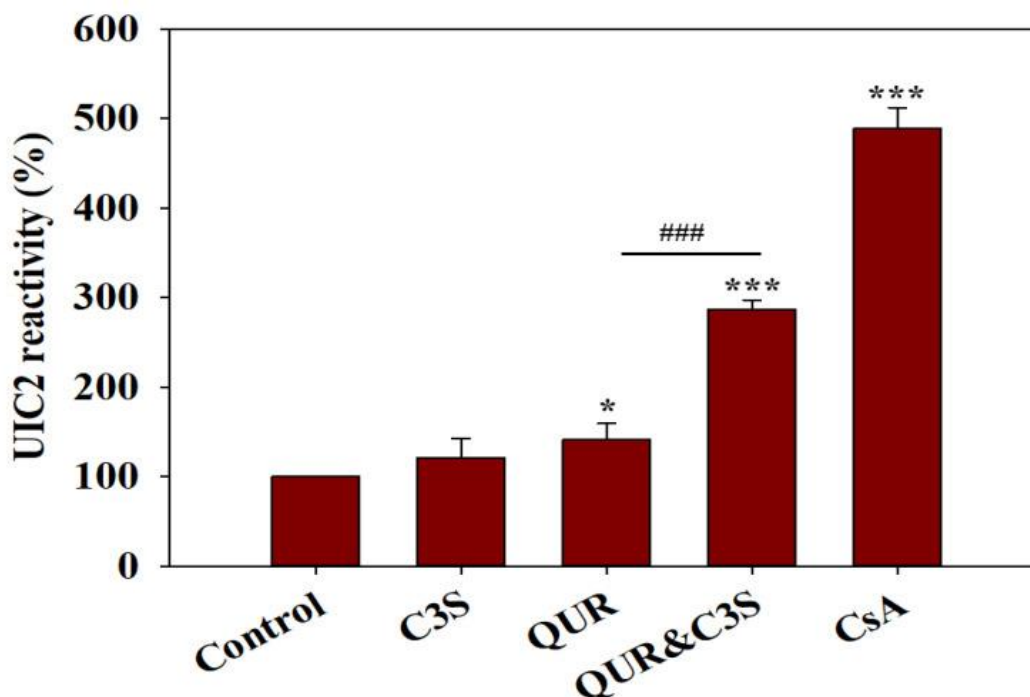


Figure 23. Effects of QUR and C3S on the UIC2 reactivity of ABCB1-positive NIH 3T3 cells. The UIC2 reactivity of polyphenol or CsA treated cells was expressed as percentage of the untreated control. Cells were pre-incubated in the presence or absence of cyclosporine A (CsA, 10 μ M), QUR, C3S or their combination for 10 min at 37 $^{\circ}$ C and then further incubated with 10 μ g/mL UIC2-A647 for another 30 min. Bars represent the means ($\pm$ SD) of 4 independent measurements. Significant differences compared to untreated samples are shown by ***: $p < 0.001$ and *: $p < 0.05$, while significant differences between QUR and QUR&C3S treatments are shown by ###: $p < 0.001$.

5.2.3. Molecular docking studies

For docking studies, we used the cryo-EM structure of human ABCB1 in complex with the inhibitor ZQU (PDB ID: 7A6F, resolution: 3.50 $\text{\AA}$). In this structure, two ZQU molecules bind to ABCB1: one molecule occupies the central drug-binding pocket (referred as site 1) and a second molecule extends into a phenylalanine-rich cavity known as the "access tunnel" (referred as site 2). Since currently there are no docking algorithms that can simultaneously dock two molecules at separate binding sites, we sequentially docked C3S and QUR to site 1 and site 2 to find their best poses with the lowest binding free energy (**Figure 24**). This sequential docking strategy was tested by docking two ZQU molecules to ABCB1 and comparing the docked poses to the cryo-EM structure of ZQU-bound ABCB1 (see **Figure 11** in Materials and Methods).

In the first step of docking experiments, QUR and C3S one by one was docked at site 1 while keeping site 2 empty. The same docking procedure was applied for site 2, while keeping site 1 empty. For instance, C3S was docked at site 1 only which showed docking score -7.4 Kcal/mol. Similarly keeping site 1 empty C3S was docked at site 2, which showed docking score -7.7 Kcal/mol. The same approach was followed for QUR, which showed docking scores -6.6 Kcal/mol at both the sites, respectively. In the next step, we docked the other ligand molecule (similar or different kind) to the empty site while the other site was kept occupied by the previously docked ligand. By testing all the possibilities how QUR and C3S can be docked to the two binding sites of ABCB1, we selected the best binding poses of the ligand combinations with the best docking scores.

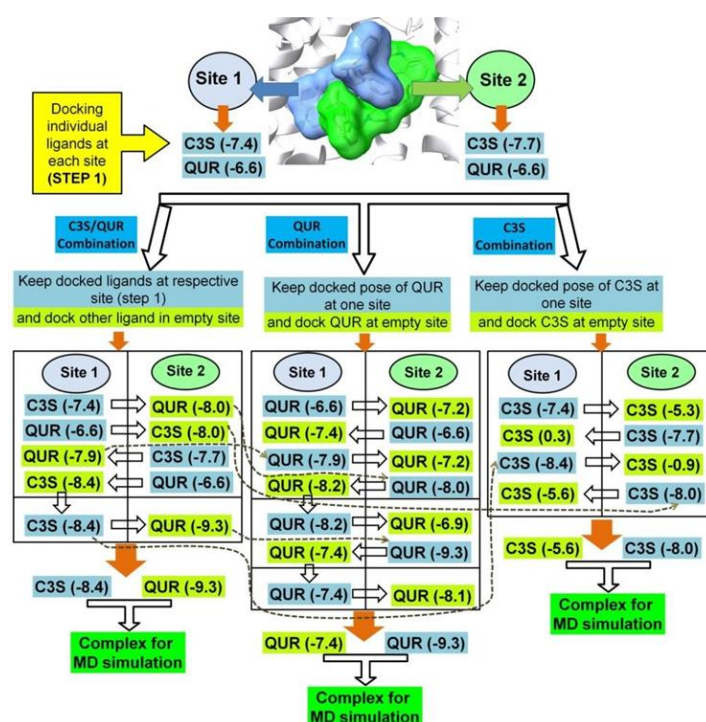


Figure 24. Docking strategy followed for docking C3S and QUR at site 1 and site 2. The text highlighted by cyan shows the name and docking score (in parenthesis) of the previously docked ligand at the respective site.

In summary, based on the docking scores, the combination of C3S at site 1 and QUR at site 2, with scores of -8.4 and -9.3 Kcal/mol respectively, appears to be the most favorable pair of inhibitors for ABCB1. The ligand-ABCB1 complex containing a QUR molecule at both sites has slightly lower docking scores (-7.4 and -9.3 respectively at site 1 and site 2), indicating potentially lower binding affinity than the combination of C3S and QUR. Interestingly, the ABCB1 complex binding two C3S molecules is the least favorable in terms of the docking

scores -5.6 and -8.0 at site 1 and site 2, respectively. The interactions of the ligands with ABCB1 in the three different complexes are shown in **Figure 25**.

Compared to ligand docking studies, MD simulations can provide more accurate insights into binding affinities and the overall stability of the protein-ligand complexes, since MD studies also take into account the flexibility of side-chain residues and the protein backbone, which cannot be considered in docking studies. Therefore, we conducted MD simulations by the help of experts (Rajesh Patil and Tamás Hegedűs) to gain further insights into the ligand-ABCB1 interactions in the above-described ligand-transporter complexes.

5.2.4. Analysis of hydrogen bonding between polyphenols and amino acids of the substrate binding pocket

Previous studies suggested that formation of hydrogen bonds may have crucial importance in the potent inhibitory effects of certain ABCB1 ligands, such as CsA, ZQU, elacridar and tariquidar [222,223]. Therefore, we analysed the hydrogen bond interactions in the three polyphenol-ABCB1 complexes. **Figure 25** shows the hydrogen bonds formed in the different complexes based on the docking experiments. However, upon MD simulations some of the hydrogen bonding interactions seemed dynamic by breaking and reforming, while others turned out to be non-stable and thus, they completely disappeared and new bonds were formed. Following the formation and breaking of hydrogen bonds during the entire length of MD simulations, we selected the hydrogen bonding interactions with highest occupancy and stability (see **Figure 26**). Based on these calculations, we identified the highest number of hydrogen bonding between the ligands and amino acid side chains of the substrate binding cavity in the 2×C3S-ABCB1 and C3S-QUR-ABCB1 complexes.

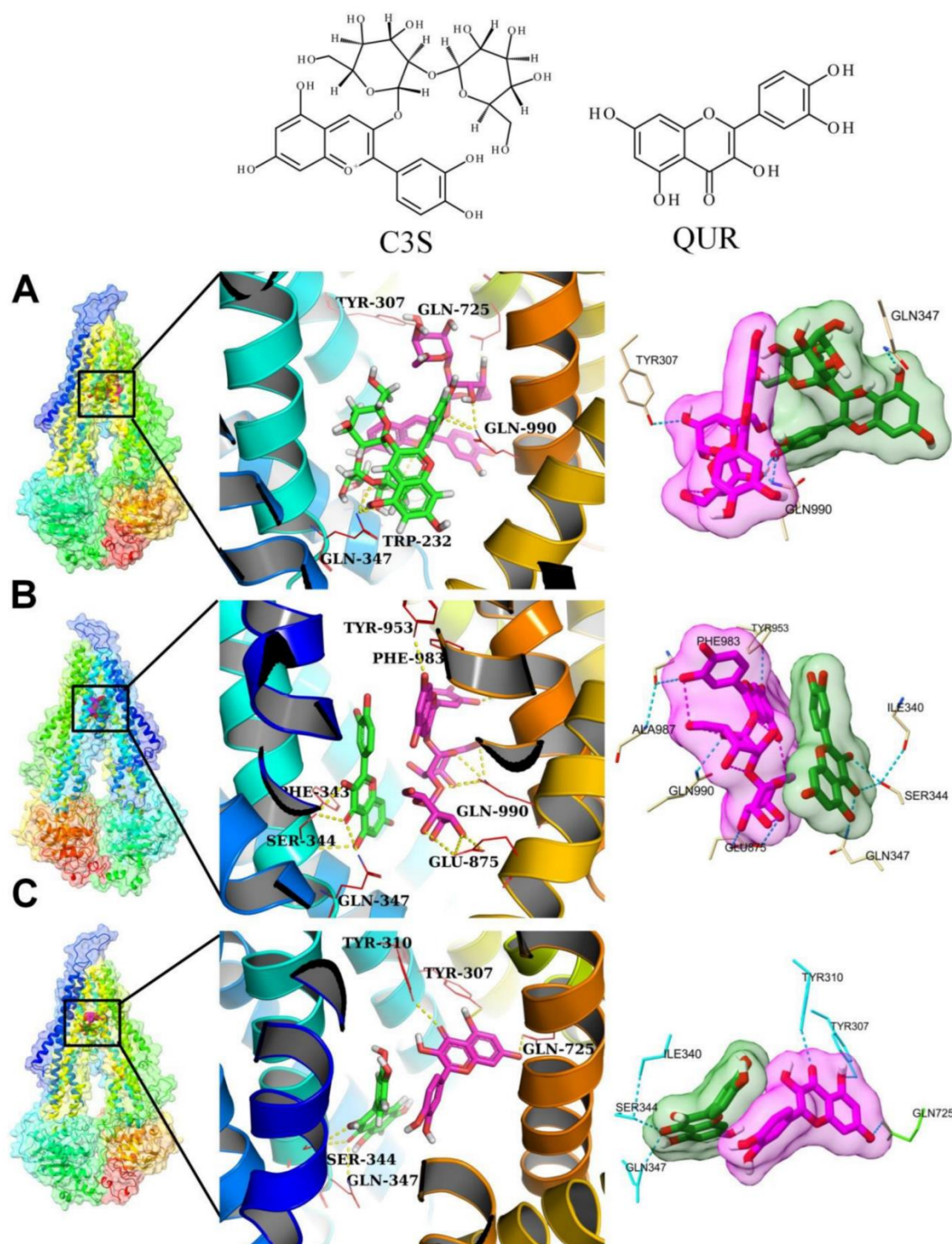


Figure 25. The best binding poses of C3S and QUR in the substrate binding pocket of ABCB1. A) ABCB1 complex with C3S at both binding sites (2×C3S-ABCB1complex); B) ABCB1 complex with C3S at site 1 and QUR at site 2 (C3S-QUR-ABCB1 complex), and C) ABCB1 complex with QUR at both sites (2×QUR-ABCB1 complex). Ligands at site 1 are shown in magenta (stick and surface representations) ligands at site 2 are depicted in green; the helices are shown in rainbow color scheme.

Interestingly, only two amino acids, namely Tyr310 and Tyr953 were identified that can form hydrogen bonds with the ligands in every studied polyphenol-ABCB1 complex. Previous studies demonstrated that these residues may have key importance in the binding of

numerous ABCB1 ligands, such as ZQU [152], tariquidar, elacridar, benzophenone sulphonamide derivatives, CsA, carvedilol and doxorubicin [223–225].

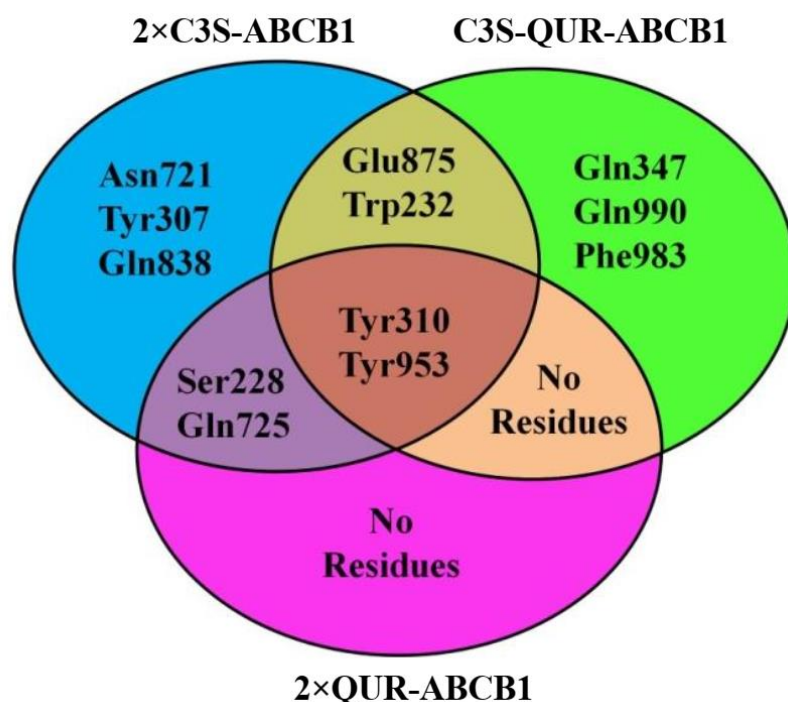


Figure 26. Venn-diagrams showing hydrogen bonding partners for C3S and QUR in the 2×C3S-ABCB1, C3S-QUR-ABCB1 and 2×QUR-ABCB1 complexes.

5.2.5. Binding free energy of the different polyphenol-ABCB1 complexes

We applied MM-PBSA calculations to assess the binding affinities of different polyphenol- ABCB1 complexes. In the MM-PBSA calculations, various energy components including van der Waals energy, electrostatic energy, polar solvation energy, and SASA energy were evaluated, and from these, the binding free energy was calculated (**Figure 27**).

The MM-PBSA results for the 2×C3S-ABCB1 complex showed that C3S at site 1 has somewhat lower binding energy ($\Delta G_{\text{bind}} = -95.413$ kJ/mol) compared to C3S at site 2 ($\Delta G_{\text{bind}} = -87.369$ kJ/mol). However, when both ligands were considered together the resulting collective binding free energy ($\Delta G_{\text{bind}} = -166.995$ kJ/mol) was significantly lower compared to that of the individual ligands.

When the 2×QUR-ABCB1 complex was subjected to MM-PBSA calculations, the binding free energies (ΔG_{bind}) for QUR at site 1 and site 2 were -73.702 kJ/mol and -84.175 kJ/mol, respectively. The combined ΔG_{bind} for this pair of ligands was -144.93 kJ/mol, which is less favorable compared to the 2×C3S-ABCB1 complex.

For the C3S-QUR-ABCB1 complex involving C3S at site 1 and QUR at site 2, it was observed that the ΔG_{bind} value for site 1 was -122.656 kJ/mol, which is significantly lower than that of C3S at site 1 in the 2×C3S-ABCB1 complex. Similarly, the ΔG_{bind} value for QUR at site 2 was -98.603 kJ/mol, which is lower than the ΔG_{bind} value of QUR at the same site in the 2×QUR-ABCB1 complex. Moreover, the cumulative ΔG_{bind} value for the C3S-QUR-ABCB1 complex was calculated to be -218.960 kJ/mol, which is the lowest among all the ligand pairs supporting that it is the most favorable ligand combination.

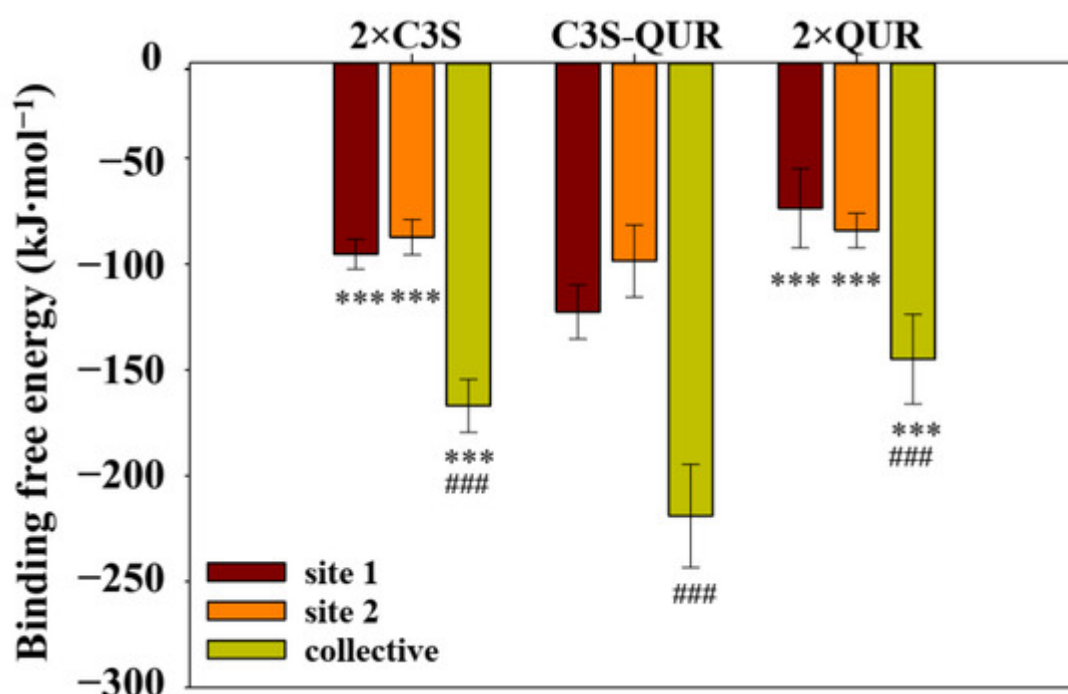


Figure 27. Binding free energies of different ligand-ABCB1 complexes determined by MM-PBSA calculations. Binding free energies were calculated in site 1, in site 2, and when both binding sites were considered together (i.e., collective) using trajectories extracted from the 300 ns to 400 ns MD time intervals with 250 ps steps. Bars represent mean values ($\pm$ SD, $n = 401$). Significant differences compared to the C3S-QUR-ABCB1 complex (***: $p < 0.001$). Significant difference of the collective binding free energy compared to the individual sites (###: $p < 0.001$).

6.1. Discussion I.

In our study, we analysed the interaction of 15 polyphenols with ABCB1 measuring the ATPase activity, substrate transport activity and UIC2 reactivity of ABCB1. Based on these experiments we discriminated polyphenols that are substrates or inhibitors of ABCB1 and those that do not have any significant effect on the function of ABCB1 (see **Table 3**).

Our experiments revealed that QUR, quercetin-3-glucoside and ellagic acid act as inhibitors of ABCB1, since they reduced both the transport and ATPase activity of the pump (**Figures 14 and 16**). It should be noted that the ABCB1 inhibitory effect of polyphenols was found weaker than that of the reference inhibitor CsA (**Figure 16**). In our hands, QUR showed the strongest ABCB1 inhibitory effects among the studied polyphenols, although it could not induce complete inhibition of ABCB1 mediated transport and ATPase activity even at high concentrations (**Figure 14 and 16**).

Table 3. Interactions of polyphenols with ABCB1

Compounds	Basal ATPase activity	Verapamil stimulated ATPase activity	Substrate accumulation	UIC2 shift	Substrate /Inhibitor
quercetin (QUR)	---	---	+++	++	inhibitor
ellagic acid	---	---	++	+	inhibitor
quercetin-3-glucoside	--	--	+	ns	inhibitor
narcissoside	--	ns	ns	ns	n/a
cyanidin-3-sophroside (C3S)	++	--	+	ns	substrate
kuromanin	+	--	ns	ns	substrate
catechin	+	-	+	ns	substrate
caffeic acid	+	-	+	ns	substrate
naringenin	+	-	+	ns	substrate
epicatechin	ns	-	ns	ns	substrate
chlorogenic acid	ns	-	ns	ns	n/a
trans-ferulic acid	ns	-	ns	ns	n/a
keracyanin	ns	+	+	ns	n/a
oenin	ns	-	ns	ns	n/a
malvin	ns	-	ns	ns	n/a

(-: decreasing effect, +: increasing effect; **ns**: not significant, **n/a**: not applicable)

On the other hand, C3S, kuromanin, catechin, caffeic acid and naringenin behaved as ABCB1 substrates, since they stimulated the basal ATPase activity of ABCB1, while

decreasing the verapamil stimulated ATPase activity (**Figure 13**). In calcein and daunorubicin accumulation experiments, these compounds generally brought about weaker increase of substrate accumulation compared to other compounds deemed inhibitors (**Figure 16**).

Besides substrate accumulation experiments and ATPase assays, we also conducted DPH and TMA-DPH anisotropy measurements in live cells to answer the question whether the tested polyphenols can change the fluidity and the packing order of the membrane. In these experiments we found that QUR and C3S modified the TMA-DPH and DPH anisotropy values of cells indicating membrane fluidity changes (see **Figure 19**). Since the duration of anisotropy measurements is quite short (30-40 min), it seems unlikely that polyphenol treatments could modify the overall lipid composition of the plasma membrane in this time range. Therefore, it seems plausible that C3S and QUR change the membrane fluidity directly by their partitioning into the plasma membrane. We observed that QUR and C3S increased the anisotropy of TMA-DPH in ABCB1-positive and ABCB1-negative cells alike, which reflects an increased ordering in the lipid head group region of the membrane in agreement with previous experiments carried out on liposomes [226,227].

Interestingly, in DPH anisotropy measurements, we found that QUR increased the membrane fluidity in ABCB1-positive cells, while decreasing it in ABCB1-negative cells (see **Figure 19B**). Previous studies suggested that the effect of certain flavonoids (e.g., kaempferol) on membrane fluidity is concentration dependent [228]. Therefore, the fluidizing effect of QUR in ABCB1-positive cells may be explained by the ABCB1-dependent sequestration of QUR in the acyl-chain region of the membrane monitored in DPH anisotropy measurements. However, further experiments are needed to clearly understand the details of this observation. In view of the fact that ABCB1 is sensitive to the alterations of membrane fluidity, the above-described membrane specific effects of QUR may also contribute to its ABCB1 inhibitory effects [229,230].

We also observed that quercetin-3-glucoside possesses weaker ABCB1 inhibitory effects compared to QUR (**Figures 14 and 16**). At the same time, we found that quercetin-3-glucoside did not affect the TMA-DPH and DPH anisotropy values (**Figure 19**), implying that it may not reach membrane concentrations sufficient to affect the packing order and the fluidity of the plasma membrane. The lower membrane partitioning of quercetin-3-glucoside is also supported by its lower octanol–water partition coefficient ($\log P_{ow}$) compared with QUR [231]. However, it should be noted that the $\log P_{ow}$ parameter is not always a good predictor for the plasma membrane partitioning of compounds [232]. In quercetin-3-glucoside, the presence of

a sugar moiety changes the shape of the molecule, and thus it may affect its interaction either with the plasma membrane or the drug-binding pocket of ABCB1.

Interestingly, catechin behaved as a low affinity substrate, while its stereoisomer epicatechin did not show any signs of interaction with ABCB1, in agreement with a previous report [233]. In fact, several publications reported stereo-selective differences in the interaction of certain ligands with ABCB1. For instance, a structural study demonstrated that cyclic oligopeptide stereoisomers QZ59-RRR and QZ59-SSS interact differently with the drug binding pocket of ABCB1: QZ59-RRR binds to ABCB1 at only one site located at the center of the drug-binding pocket, while QZ59-SSS occupies two distinct sites [124]. Our research group also observed previously that the recognition of certain retinoids by ABCB1 and ABCG2 is also stereo-selective [218].

C3S and kuromanin showed a bell-shaped ATPase stimulation curve (**Figure 13**). The same tendency was observed for several previously identified ABCB1 substrates, such as verapamil (see also **Figure 13F**) or vinblastine [200,234,235]. This concentration dependence is supposed to reflect the gradual saturation of the substrate-binding pocket of ABCB1. The interaction of ligands (substrates, inhibitors) with ABCB1 may occur on a modular basis: ligands bind to only one site at lower concentrations, while occupying two or more sites simultaneously at higher concentrations [199]. In agreement with this hypothesis, several drugs (e.g., azidothymidine, paliperidone, abacavir) that are efficiently transported by ABCB1 become potent inhibitors when two identical molecules are chemically cross-linked, supporting that substrates may transform into inhibitors when two molecules of them bind simultaneously to the drug-binding pocket [236,237].

We hypothesized that by combining different ABCB1 ligands more pronounced transporter inhibition could be reached. Therefore, we also investigated the effect of QUR, naringenin and ellagic acid in combination with verapamil on the activity of ABCB1. We observed that the transport inhibitory effect of the polyphenol–verapamil combinations is additive or synergistic (**Figure 20**). Interestingly, when QUR was applied together with 40 μ M verapamil, a synergistic increase of calcein accumulation was observed in ABCB1-positive cells up to the level of ABCB1-negative cells (**Figure 20A**). Similar synergistic ABCB1 inhibitory effect was seen, when QUR was combined with C3S (**Figures 21 and 22**). In addition, we detected a strong increase in UIC2 reactivity in response to QUR–verapamil (**Figure 20G**) or QUR-C3S (**Figure 23**) treatments, supporting the idea that the combined treatments stabilize the UIC2 reactive inward-facing conformation of ABCB1. Although the exact mode of inhibitor binding to ABCB1 and the action mechanism of potential inhibitors is

not known, it seems likely that numerous potent inhibitors act by stabilizing the inward-facing conformational state of the pump [16,50] and consequently hinder the formation of the NBD sandwich dimer and thus inhibit ATP hydrolysis.

The observed additive and synergistic ABCB1 inhibitory effects may call the attention to the potential risks of drug–drug interactions associated with the consumption of dietary polyphenols concurrently with chemotherapy treatments involving ABCB1 substrate/inhibitor drugs like verapamil. Since a diverse range of polyphenols are proved to be present at high amounts in fruits, vegetables, coffee and tea, even weak ABCB1 inhibitory effects of the individual compounds can be amplified by the possible additive or synergistic interactions between them at the level of ABCB1.

6.2. Discussion II.

In the second part of our study, we have analysed the synergistic ABCB1 inhibitory effect of the co-treatment with C3S and QUR in more detail. To study the interaction of C3S and QUR with the substrate binding pocket of ABCB1, we carried out *in silico* ligand docking and MD simulation experiments. Our results support that C3S and QUR may bind simultaneously to the large substrate-binding pocket of ABCB1. According to our docking studies the most favourable binding poses were obtained when the bulkier C3S molecule bound to the central substrate binding site and the smaller QUR molecule occupied the “access tunnel”. Our MD simulations also demonstrated that the simultaneous binding of two C3S or two QUR molecules to the large substrate-binding pocket of ABCB1 is energetically less favourable compared to the C3S–QUR combination (**Figure 27**). However, it should be noted that the binding energy differences alone cannot explain the stronger inhibitory effect of the C3S–QUR combination. Interestingly, we also observed that co-treatment with C3S and QUR strongly increases the UIC2 reactivity, suggesting that this ligand combination can stabilize the UIC2-reactive inward-facing conformation of ABCB1 similar to the potent inhibitor CsA (**Figure 23**). It seems plausible that the larger number and higher occupancy of the hydrogen bonds formed in case of the C3S-QUR combination compared to the simultaneous binding of two QUR molecules may contribute to the conformational stabilization of the transporter (see **Figure 26**). Although we identified comparable number of hydrogen bonds between ligands and amino acid residues lining the substrate binding pocket in the C3S-QUR-ABCB1 and 2×C3S-ABCB1 complexes (**Figures 25 and 26**), the 2×C3S-ABCB1 complex turned out to be less favorable energetically (**Figure 27**) probably due to steric constraints imposed by the bulkier C3S molecules.

Taken together, combined application of C3S and QUR may result in their simultaneous binding to the substrate binding cavity, as it was observed in our *in silico* studies (**Figures 25 and 26**), leading to stabilization of the UIC2 reactive inward facing ABCB1 conformation (**Figure 23**) and complete inhibition of the transport and ATPase activities of ABCB1 (**Figures 21 and 22**). QUR showed only partial ABCB1 inhibition even at high concentrations that may be explained by the lower number of hydrogen bonds and the least favorable collective binding energy of the 2×QUR-ABCB1 complex (**Figures 26 and 27**). When applied alone, C3S stimulated the basal ATPase activity of ABCB1 with some decline of the effect at high concentrations (**Figure 13**) supporting that it interacts with the transporter as a substrate. We may speculate that for inhibition of ABCB1 both substrate binding sites should be occupied by a ligand molecule, that does not happen at lower C3S concentrations, and it may occur with very low frequency even at high concentrations.

Substrate or inhibitor binding to the substrate binding cavity of ABCB1 may induce different changes in the protein resulting in stimulation or inhibition of ATP hydrolysis in the NBSs. In line with this hypothesis a recent study using hydrogen deuterium exchange mass spectrometry (HDX-MS) demonstrated that ligands stimulating or inhibiting the ATPase activity of ABCB1 differentially modulate the dynamics of the NBDs and certain intracellular helices that are far from the substrate binding cavity [238]. However, the details of this intramolecular communication process are still largely unknown [39]. The detailed understanding of them may require thorough mutagenesis studies of amino acid residues involved in interdomain communication and new *in silico* tools, as well.

7. Summary

ABCB1 is an active transporter physiologically expressed in pharmacologically important tissue barriers and frequently associated with the development of multidrug resistance in cancer cells. Dietary polyphenols involve a chemically related, but extremely diverse group of compounds abundant in plant derived food. Previous research demonstrated that several polyphenols are substrates or inhibitors of certain ABC transporters.

Our experiments revealed that certain dietary polyphenols found in sour cherry and other red berries interact with human ABCB1 as substrates or inhibitors. When applied alone, these polyphenols did not induce complete ABCB1 inhibition. However, combined treatments with low concentration of verapamil and various polyphenols such as QUR, naringenin or ellagic acid brought about a practically complete inhibition of ABCB1. Similarly, the combined application of QUR and C3S also induced a synergistic ABCB1 inhibitory effect, which could be exploited in chemotherapy protocols targeting multidrug-resistant tumors.

To understand the molecular details of the interaction of C3S and QUR we carried out *in silico* ligand docking studies and MD simulations. These experiments revealed that C3S and QUR may bind simultaneously to the complex substrate-binding pocket of ABCB1. The most favourable binding poses were obtained when the bulkier C3S molecule bound to the central substrate binding site and the smaller QUR molecule occupied the “access tunnel”. Binding free energy calculations demonstrated that the simultaneous binding of two C3S or two QUR molecules to the complex substrate-binding pocket of ABCB1 is energetically less favourable compared to the C3S–QUR combination.

The additive or synergistic transport inhibitory effects observed with polyphenol–verapamil combinations highlight a potential risk of drug–drug interactions on the level of ABCB1. These interactions necessitate more caution when consuming dietary polyphenols alongside chemotherapy regimens involving ABCB1 substrate or inhibitor drugs.

Collectively, the integration of wet lab data and *in silico* studies enhances our understanding of the molecular mechanisms underlying ligand–ABCB1 interactions and pave the way for more efficient inhibition strategies of ABCB1 to overcome multidrug resistance.

8. References

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9. List of Publications



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PhD Publication List

Candidate: Kuljeet Singh

Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. Singh, K., Patil, R. B., Patel, V., Gálné Remenyik, J., Hegedűs, T., Goda, K.: Synergistic Inhibitory Effect of Quercetin and Cyanidin-3O-Sophoroside on ABCB1.
Int. J. Mol. Sci. 24 (14), 1-24, 2023.
DOI: <http://dx.doi.org/10.3390/ijms241411341>
IF: 5.6 (2022)
2. Singh, K., Tarapcsák, S., Gyöngy, Z., Ritter, Z., Batta, G., Bosire, R., Gálné Remenyik, J., Goda, K.: Effects of Polyphenols on P-Glycoprotein (ABCB1) Activity.
Pharmaceutics. 13 (12), 1-17, 2021.
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List of other publications

3. Sharma, G., Sharma, D., Singh, K., Jha, P., Bansal, N., Mishra, Y. K., Singh, K., Sharma, A., Sharma, S. K.: Synthesis of biogenic calcium silicate glasses from biomass: Physical, dielectric and electrical properties.
J. Non-Cryst. Solids. 612, 1-8, 2023.
DOI: <http://dx.doi.org/10.1016/j.jnoncrysol.2023.122336>
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10. Keywords

Multidrug resistance, ABC transporter, ABCB1 (P-glycoprotein, Pgp), polyphenols, ATPase activity, UIC2 reactivity, membrane fluidity, substrate recognition, molecular docking.

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12. APPENDIX