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Effects of polyphenols on P-glycoprotein (ABCB1) activity

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1. INTRODUCTION, LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

ABCB1 (P-glycoprotein/MDR1) is an ABC family membrane transporter that uses ATP to expel diverse lipophilic and amphiphilic compounds, including xenobiotics, metabolic by-products, and many anticancer drugs from cells. It is expressed in pharmacological barriers (e.g., intestine, brain, placenta, testis) and detoxifying organs like liver and kidneys. 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.

Chemotherapy is a key therapeutic modality for treating systemic cancers like leukaemia, lymphoma, and metastases. However, tumors often develop multidrug resistance (MDR), i.e., cross-resistance to various anticancer agents. This arises from changes in drug targets like cell cycle proteins, DNA repair enzymes and apoptotic pathways, or from reduced drug accumulation due to ABC transporter overexpression (e.g., ABCB1, ABCG2, ABCC1). MDR causes ~50% of chemotherapy failures up to 90% in metastatic cases. Thus, understanding ligand–ABC transporter interactions is vital for designing strategies to combat resistance.

1.2 ROLE OF ABC TRANSPORTERS IN MULTIDRUG RESISTANCE – A HISTORICAL OVERVIEW

Before ABC transporters were characterized, theories explaining MDR in cancer emerged. Goldstein et al. described AD-resistant HeLa cells surviving high actinomycin D (AD) doses. Later, Biedler's AD-resistant hamster cells showed cross-resistance to vincristine and daunorubicin (DNR), supporting a membrane-based resistance mechanism. Dano found that DNR-resistant cells had reduced drug accumulation, while nuclei from wild-type and resistant cells absorbed DNR similarly suggesting membrane transport changes. Structural analogs and metabolic inhibitors enhanced DNR uptake, and co-administered drugs (e.g., vinblastine) blocked resistance via competitive inhibition. This transporter was later identified as P-glycoprotein (Pgp) by Ling. Gros et al. cloned the drug-resistance gene from hamster cells, and it conferred MDR when transfected into sensitive cells. The human gene was cloned in 1986 and named MDR1 (now referred as ABCB1) its expression correlated with resistance, and its transfection induced MDR. Later findings confirmed that other ABC transporters such as ABCG2 and ABCC1 can also mediate MDR. MRP1 (ABCC1), discovered by Cole et al. in 1992, confers resistance to many drugs (e.g., vincristine, paclitaxel, methotrexate). It typically exports glutathione, glucuronide, or sulphate conjugates, acting as an organic anion transporter. ABCC1 also recognizes some newer anticancer agents that target tumor proliferation pathways. ABCG2 (also called BCRP, MXR, or ABCP) was identified

independently by three groups. It transports hydrophobic/amphipathic chemotherapeutics and endogenous toxic products like pheophorbide A and uric acid.

1.3 GENERAL STRUCTURE OF ABC PROTEINS

ABC proteins are a large superfamily found in all organisms from Archaea to humans. Humans have 48 ABC proteins, categorized into 7 subfamilies (ABCA–ABCG) based on sequence and structure. Most of them function as active transporters; others act as ion channels or regulators. Subfamilies E and F lack transmembrane domains (TMDs) and are involved in mRNA splicing or transcription complexes.

Functional ABC transporters typically contain two cytoplasmic nucleotide-binding domains (NBDs) that hydrolyze ATP and two TMDs that recognize and translocate substrates. The conserved NBDs consist of a core present in many ATPases and a signature sequence unique to ABC proteins. These form composite catalytic sites with ATP bound between Walker A/B motifs of one NBD and the signature sequence of the other. Walker A (G-x(4)-GK-[TS] binds ATP phosphates, while Walker B coordinates Mg^{2+} and hydrolyzes ATP, with the "catalytic glutamate" being critical. Some ABC proteins, like CFTR and SUR1/2 (ABCC8/9), have one degenerate NBS that poorly hydrolyzes ATP.

Unlike conserved NBDs, TMDs vary structurally depending on function. In lower organisms, ABC proteins can be importers or exporters, while human ABCs are mostly exporters. Importers move substrates toward the cytosol, while exporters move them outward. Human exporters are classified into type I and II. Type I (e.g., ABCB1) shows "domain swapping," where each TMD includes helices from both transporter halves. Type II transporters (e.g., ABCG2) lack this feature. Some ABCs also have accessory domains like extracellular domains (ECDs) in ABC proteins or an extra TMD in some ABCB and ABCC members.

1.4 PHYSIOLOGICAL FUNCTIONS AND DISORDERS ASSOCIATED WITH HUMAN ABC PROTEINS

Several human ABC transporters move lipophilic molecules like phospholipids, cholesterol, and phytosterols across membranes. ABCA1 exports excess cholesterol and phospholipids to apolipoproteins (apoA1, apoE), forming HDL; mutations can cause Tangier disease. ABCA12 transports lipids such as ceramides in epidermal cells; mutations cause Harlequin ichthyosis, impairing skin barrier and increasing infection risk. ABCD1 transports very long chain fatty acids into peroxisomes; its dysfunction causes adrenoleukodystrophy (ALD).

Mutations in ABCG5/G8 hinder phytosterol excretion, causing sitosterolaemia and atherosclerosis. ABCC6 exports nucleotides like ATP; its mutation leads to Pseudoxanthoma elasticum

(PXE), marked by ectopic tissue calcification. ABCG2 is vital for intestinal uric acid elimination. Its mutations like 141K reduce cell surface expression, contributing to hyperuricemia and gout.

Other ABC transporters affect the composition of bile by taking part in the excretion of several bile components. For example, ABCB11 (BSEP) exports bile salts, and its mutation causes familial intrahepatic cholestasis type II.

ABCC7 (CFTR) regulates Cl^- transport in epithelial cells. Certain mutations of ABCC7 cause cystic fibrosis (CF), a lethal disease with mucus accumulation and lung infections.

ABCC8 and ABCC9 form ATP-sensitive K^+ channels (KATP) with Kir6 subunits. ABCC8 mutations link to hyperinsulinism and neonatal diabetes, while ABCC9 is tied to cardiovascular and neurological diseases.

1.5 EXPRESSION PROFILE AND PHYSIOLOGICAL FUNCTION OF HUMAN ABCB1

ABCB1 actively exports diverse range of lipophilic and amphiphilic compounds from cells, including both exogenous and endogenous toxins. It is localized on the apical membrane of cells in various barriers, including the intestinal epithelium, blood-brain barrier, hepatocyte biliary membrane, adrenal glands, kidney proximal tubules, Sertoli cells, and placental trophoblasts. Notably, ABCB1 is also expressed in fetal membranes, helping shield the developing embryo from toxins. Its broad tissue distribution and substrate range indicate its key role in eliminating xenobiotics via urine, bile and the intestinal tract, and in preventing their accumulation in sensitive organs like the brain and testis. Moreover, ABCB1 significantly influences the ADME-Tox profile of many chemotherapeutic agents.

Although Abcb1a knockout mice and Collie dogs lacking ABCB1 show no overt phenotype, they experience neurotoxicity when exposed to ABCB1 substrates like ivermectin and vinblastine, emphasizing its role in CNS protection. Additionally, ABCB1 is expressed in hematopoietic stem cells and drug-tolerant persistent cancer cells, where it contributes to cellular defense mechanisms.

1.6 STRUCTURE OF HUMAN ABCB1

The human ABCB1 protein comprises 1,280 amino acids (molecular weight is ~170 kDa) and includes two transmembrane domains (TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2). Each TMD contains 6 transmembrane α-helices linked to an NBD. The two halves are connected by a flexible “linker” region (residues 627–688), rich in phosphorylation sites and likely

involved in regulation. TMDs are connected to NBDs via intracellular loops (ICLs) and non-covalently through intracellular coupling helices (ICHs), which interact with the Q- and X-loops of the NBDs.

Due to “domain swapping,” helices form two interleaved bundles creating a large internal cavity for substrate binding and transport. ABCB1 undergoes conformational changes during its transport cycle: the inward-facing (IF) state forms a central cavity accessible from the cytoplasm, while the outward-facing (OF) state brings the NBDs close and opens the cavity to the extracellular space, facilitating substrate release. Additionally, occluded conformations, where both cytoplasmic and extracellular access are closed, have been observed. These likely represent intermediates in the transport cycle. Cryo-EM and X-ray structures of these conformations serve as crucial templates for mutagenesis, functional assays, and molecular dynamics (MD) simulations exploring ABCB1 mechanisms.

1.7 TRANSMEMBRANE DOMAINS AND THEIR ROLE IN SUBSTRATE BINDING

ABCB1 is thought to have evolved from an ancient half-transporter with one NBD and one TMD through gene duplication and mutation, resulting in a pseudo-symmetric structure. Comparative analysis in mice showed higher conservation between the two NBDs (77.3% similarity) than between the TMDs (43.5%). Together, the TMs form a ~6,000 $Å^3$ central binding cavity, composed of TM1, 4–6 from TMD1 and TM7, 10–12 from TMD2, lined with aromatic, hydrophobic, and some polar/charged residues.

Though the exact mechanism of substrate recognition remains unclear, evidence from photoaffinity labeling with [^{125}I]iodoaryl azidoprazosin supports the presence of two non-identical drug-binding regions. Functional studies proposed three substrate sites: H (Hoechst 33342/colchicine), R (rhodamine 123/anthracyclines), and M (progesterone/prazosin), which may exert allosteric effects on each other. Molecular docking confirmed that all three sites can accommodate common ABCB1 substrates like verapamil and doxorubicin.

Radioligand binding assays suggested at least four distinct or overlapping binding sites based on competitive and non-competitive interactions. Cysteine mutagenesis and thiol-reactive substrate studies indicated that many drugs (e.g., cyclosporine A (CsA), tariquidar, valinomycin) share overlapping binding zones. This supports the “substrate-induced fit” model, where ABCB1 adapts its binding pocket via conformational changes to accommodate diverse molecules.

X-ray and cryo-EM studies further confirmed that ligands bind to partially overlapping sites in the central cavity. Notably, inhibitors such as elacridar and zosuquidar often bind in pairs: one at the central pocket and the other in an adjacent "access tunnel" near TM4 and TM10. These dual interactions stabilize the protein in an inactive state by preventing NBD dimerization and ATPase activity.

1.8 TRANSPORT MODELS EXPLAINING THE WORKING MECHANISM OF MEMBRANE TRANSPORTERS

Three main models describe membrane transporter mechanisms. The “pump model” posits that substrates bind in the cytoplasmic aqueous phase, cross the lipid bilayer via the transporter, and are released into the extracellular fluid. This model best explains the transport of hydrophilic compounds with poor membrane permeability.

However, ABCB1 typically transports amphiphilic or lipophilic substrates (e.g., verapamil) that have high logPow values and readily accumulate in the membrane at concentrations far exceeding those in the aqueous phase. The “hydrophobic vacuum cleaner model” was proposed based on this observation, where drugs partition into the inner membrane leaflet, from where ABCB1 expels them into the extracellular space. This model is supported by fluorescence and EPR studies with labeled verapamil.

The “flippase” model, now more accurately termed the “floppase” model, suggests that ABCB1 captures substrates from the inner leaflet and moves them to the outer leaflet, where they may either diffuse out or flip back for recapture. Both this and the vacuum cleaner model agree that drug binding primarily occurs at the cytoplasmic leaflet.

A nice experimental support comes from proteo-liposome studies where Omote and Al-Shawi showed that a charged verapamil derivative, unable to passively diffuse, was transported from the outer to inner leaflet (due to inverted ABCB1 orientation), demonstrating leaflet-specific transport. This led to verapamil accumulation and surface charge changes.

In summary, ABCB1 likely translocates substrates from the inner leaflet to the outer leaflet or extracellular space, with the precise pathway depending on substrate hydrophobicity and partitioning characteristics.

1.9 MEMBRANE PHYSIOCHEMICAL PROPERTIES MODULATE THE FUNCTION OF ABCB1

As an integral membrane protein, ABCB1 resides in the complex lipid-protein matrix of the plasma membrane, where lipid diversity (in head groups, acyl chains, and saturation) critically affects its structure and function. Membrane properties such as lateral pressure, thickness, and fluidity influence ABCB1's conformational dynamics and activity. Studies have shown that changes in membrane fluidity and composition modulate ABCB1 function.

In addition, cryo-EM analysis has identified a “lipid belt” around ABCB1's transmembrane helices with cholesterol in the outer leaflet and phosphatidylethanolamines/cholesterol in the inner leaflet. Removing this annular lipid layer via detergents inactivates ABCB1 and related transporters.

ABCB1 activity is also influenced by substrate availability. Lipophilic/amphipathic drugs often accumulate in the membrane, reaching millimolar concentrations, leading to low affinity but efficient binding. This behaviour supports the polyspecificity of ABCB1.

In both the hydrophobic vacuum cleaner and flippase models, prolonged residence time of substrates in the inner leaflet promotes their interaction with ABCB1. Membrane lipid composition and lateral packing density critically modulate this process. For example, doxorubicin’s flip-flops across the bilayer is inhibited by increased cholesterol content, but accelerated by heat and benzyl alcohol.

1.10 INTERACTIONS OF POLYPHENOLS WITH ABCB1

Polyphenols are plant-derived secondary metabolites, with over 9,000 identified compounds. They play important roles in plant defense and reproduction. Structurally, they are grouped into phenolic acids, flavonoids, stilbenes, and lignans. Phenolic acids, known for their antioxidant activity, include hydroxybenzoic acids (e.g., ellagic acid) and hydroxycinnamic acids (e.g., caffeic, chlorogenic, and ferulic acid). Flavonoids, the most abundant dietary polyphenols, contain a chromone ring with varied side chains, forming subclasses such as flavans (catechin, epicatechin), flavones (apigenin, luteolin), flavonols (quercetin), flavanones (naringenin), chalcones, and anthocyanins (cyanidin derivatives, like cyanidin-3O-sophoroside (C3S), oenin, malvin, kuromanin).

Polyphenols are prevalent in fruits, vegetables, flowers, coffee, and tea, offering antioxidant, antiviral, and anti-inflammatory benefits, and are linked to protection against cancer, cardiovascular, and neurodegenerative diseases. However, food-drug interactions have been reported, especially with polyphenol-rich foods like grapefruit juice, which affects drugs such as calcium channel blockers and midazolam. Such interactions may occur through effects on cytochrome P450 enzymes, membrane transporters, or receptors, altering drug pharmacokinetics and efficacy. ABCB1, being a key efflux transporter of chemotherapeutics, is a likely target for such interactions.

Studies show that several polyphenols including nobiletin, auraptene, sesamin, ginkgolic acid, matairesinol, glycyrrhetinic acid, glabridin, phyllodulcin increase daunorubicin accumulation in ABCB1-expressing KB-C2 cancer cells. While auraptene and nobelitin stimulated, glycyrrhetinic acid inhibited verapamil-stimulated ABCB1 ATPase activity. Ellagic acid has also been shown to inhibit ABCB1.

Some polyphenols enhance anticancer drug efficacy in MDR cells by inhibiting ABCB1, ABCC1, and ABCG2. For example, karanjin is a broad-spectrum ABC transporter inhibitor, more potent than apigenin, genistein, or naringenin. Licochalcone A, from licorice root, interacts with ABCG2 inhibiting its transport activity and stimulating its ATPase activity. Chrysin and retusin also inhibit ABCG2 function.

Thus, polyphenols represent a natural source of potential ABC transporter inhibitors. Understanding their interactions with transporters like ABCB1 may also help prevent unwanted food-drug interactions at the transporter level.

2. 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 (QUR) and cyanidin-3O-sophoroside (C3S) with ABCB1 by using transport and ATPase assays and *in silico* ligand docking experiments.

3. MATERIALS AND METHODS

3.1 CHEMICALS

All chemicals, cell culture media and supplements used for the study were purchased from Sigma-Aldrich (Budapest, Hungary). Fluorescent dyes including calcein-acetoxymethylester (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 using affinity chromatography. The antibody-producing hybridoma cell lines were obtained from the American Type Culture Collections (Manassas, VA, USA). UIC2 and 15D3 mAbs were labeled with A647. The Ab dye conjugates were separated 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 hydrophilic compounds such as verapamil were dissolved in distilled water. All tested polyphenols (quercetin, quercetin-3-glucoside, naringenin, ellagic acid, cynidin-3O-sophroside, 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 water according to the manufacturer's instructions. For all experiments, the final DMSO concentration of samples was less than 1% (v/v).

3.2 CELL LINES

The NIH 3T3 mouse fibroblast cell line and its human ABCB1 overexpressing counterpart, NIH 3T3 MDR1 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).

3.3 HARVESTING CELLS FROM CULTURE FLASKS

We used trypsin–EDTA solution (0.25 % trypsin-EDTA in PBS) to prepare cell suspension from adherent cell cultures. First the culture media was completely removed from the culture flask. For a T-75 culture flask, cells were rinsed 3 times with 2 ml of cold PBS to remove cell debris and culture media. 2 ml of trypsin–EDTA was then added and the flask transferred to a 37°C water bath for 2-3 minutes. Detachment of cells was facilitated by gently tapping the side of the flask. 4 ml of cold PBS with 10 % FBS was added to stop the action of trypsin and then the cells were centrifuged at 435 × g for 5 minutes. The supernatant was discarded and the cells were washed twice with PBS and then re-suspended in cold PBS containing 7 mM glucose (gl-PBS).

3.4 SUBSTRATE ACCUMULATION TESTS

To study the effect of polyphenols on the transport activity of ABCB1, we measured the intracellular accumulation of fluorescent ABCB1 substrates calcein-AM and daunorubicin. Samples containing 0.5×10^6 cells/mL were pre-treated with 10 μM CsA, 40 μM verapamil and/or the tested polyphenols (10-150 μM) for 10 min at 37 °C and then further incubated with 3.8 μM daunorubicin for 30 min or 0.5 μM 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.

3.5 UIC2 AND 15D3 REACTIVITY MEASUREMENTS

The ABCB1-positive NIH 3T3 MDR1 cells (5×10^5 cells/ml in gl-PBS) were pre-treated with 10 μM CsA, 40 μM verapamil and/or the tested polyphenols for 10 min at 37°C. Subsequently, the ABCB1-specific monoclonal antibodies UIC2-A647 (10 μg/mL) or 15D3-A647 (30 μg/mL) were added to the samples and then they were further incubated at 37°C for an additional 30 min. After incubation the samples were washed twice with ice-cold gl-PBS containing 1 % FBS and centrifuged for 5 min at 435 × g at 4 °C. The UIC2-A647 fluorescence intensity of the cells was measured by flow cytometry.

3.6 MEMBRANE PREPARATIONS FROM NIH 3T3 CELLS

For ATPase activity measurements we used crude membrane samples derived from ABCB1-positive and negative NIH 3T3 cells prepared according to Sarkadi's method with slight modifications. To avoid degradation of membrane proteins all steps of the preparation procedure were carried out at 4 °C in the presence of protease inhibitors. Cell homogenization was performed in TMEP solution (50 mM

Tris-HCl (pH = 7.0), 50 mM mannitol, 2 mM EGTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail (PIC, Sigma-Aldrich, Budapest, Hungary) using a glass-Teflon tissue homogenizer. After cell homogenization, intact cells and cell nuclei were sedimented at 500 × g for 10 min at 4 °C and discarded, while the resulting supernatant was kept and further centrifuged at 12000 × g for 60 min at 4 °C to pellet membrane fragments. The resulting pellet containing membrane fragments was re-suspended in TMEP solution and was stored at −70 °C until use. The protein concentration of the membrane samples was determined by the Lowry method.

3.7 ATPASE ACTIVITY MEASUREMENTS

The ABCB1-specific ATPase activity of the membrane samples was determined by measuring the amount of inorganic phosphate (Pi) released upon ATP hydrolysis. Other ATPases abundant in cells were blocked by inhibitors, such as Na-azide for the F0F1 ATPases, ouabain for Na^+/K^+-ATPase and EGTA for the Ca^{2+}-ATPases. 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 Na_3VO_4 (vanadate) for 5 min at 37 °C. After 5 min, 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, then the samples were incubated with 105 μL color reagent at room temperature (22℃) 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 2.5ml of 1% ascorbic acid. 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 of vanadate positive and negative sample pairs.

3.8 MEASUREMENT OF MEMBRANE FLUIDITY

The effects of polyphenols on the membrane fluidity were studied in ABCB1 expressing 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 thermostatic 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 detecting the vertically and horizontally polarized components of the fluorescence intensities. There is an inverse correlation between membrane fluidity and fluorescence anisotropy: the lower the anisotropy value, the higher the membrane fluidity; hence, the increase of fluorescence anisotropy is indicative of lower fluidity and higher structural order within the membrane.

3.9 FLOW CYTOMETRY

Calcein accumulation measurements were carried out on a 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 was 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).

3.10 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 ± SD. Comparison of two groups was carried out 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 $P < 0.05$.

3.11 MOLECULAR DOCKING

The cryo-electron microscopy-based (cryo-EM-based) structure of human ABCB1 in complex with the ligand zosuquidar ((ZQU), PDB ID: 7A6F, Resolution: 3.50 Å) was used for molecular docking studies. Amino acid residues not visible in the cryo-EM structure (1-31, 86-103, 631-693 & 1277-1280) were modelled using the Modeller 9.25. During 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. The correct protonation states to each residue were assigned using PROPKA program. The 2D structures of 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 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. 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. 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 in the cryo-EM structure of the 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 center are appropriate for further docking simulations.

Similarly, C3S and QUR were docked at each site with the same parameters, which 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***. 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.

4. RESULTS

SECTION I

4.1 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 and therefore this cell line is convenient for identifying potential ABCB1 substrates or inhibitors. We have analyzed the effects of 15 dietary polyphenols on the ATPase activity of ABCB1 using membrane samples prepared from NIH 3T3 MDR1 cells. 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. In control experiments we titrated the concentration of verapamil and observed that 40 μM verapamil induced the maximum stimulation of the ABCB1-specific ATPase activity. Therefore, verapamil was applied at 40 μM concentration in all experiments, when the effect polyphenols was tested on the so-called verapamil stimulated ATPase activity. Polyphenols were categorized into three subgroups based on their behavior in ATPase measurements.

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. Caffeic acid and catechin had weaker effect on the ATPase activity compared to the others. It is interesting to note that all these compounds with similar effect on ABCB1 activity belong to different sub-groups of flavonoids. 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, while narcissoside had weaker effect that did not show concentration dependence. According to general experience, compounds that slow down the rate of basal ATPase activity and inhibit substrate stimulation are ABCB1 inhibitors that are not transported by ABCB1.

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. Notably, epicatechin, which is a stereoisomer of catechin, also belongs to this inactive subgroup of polyphenols. Oenin and malvin slightly decreased the verapamil-stimulated ATPase activity of ABCB1 at high concentrations, while keracyanin induced a slight increase, when it was applied at 10 to 50 μM concentrations.

4.2 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 also increased the cellular accumulation of the fluorescent calcein and daunorubicin 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. In contrast, quercetin-3-glucoside showed weaker effects, consistent with its lower inhibitory effect observed in ATPase assays. Ellagic acid and naringenin increased the intracellular accumulation of calcein by 200–250% compared to the control, in agreement with previously reported data. However, no significant effects of ellagic acid, naringenin, or caffeic acid were observed on the intracellular accumulation of daunorubicin. 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. Interestingly, C3S, a close relative of keracyanin (belonging to the anthocyanin 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 in accordance with its modest effect on the ATPase activity.

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. There is one exception, kuromanin affected both the basal and the verapamil-stimulated ATPase activities of ABCB1, but it did not show significant effects in calcein and daunorubicin assays.

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, 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.

4.3 EFFECTS OF POLYPHENOLS ON THE UIC2 REACTIVITY OF ABCB1

Previous studies suggested that efficient ABCB1 inhibitors such as CsA and valspodar can stabilize ABCB1 molecules in an inward-facing (IF) conformation that is recognized by the conformation

selective UIC2 monoclonal antibody. 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. Our measurements demonstrated that QUR induced a significant, concentration-dependent increase in UIC2 reactivity, consistent with its strong ABCB1 inhibitory effect in ATPase and substrate accumulation assays. Ellagic acid also increased UIC2 reactivity, albeit no concentration-dependence was detected. 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.

4.4 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. There are numerous observations supporting that 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 reaching 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 and TMA-DPH.

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 that prefers the lipid/water interface of the membrane. Therefore, using TMA-DPH and DPH the membrane 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. 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.

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, 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. The measured anisotropy values did not show significant differences between the untreated ABCB1-positive and ABCB1-negative cells, which is consistent with previous findings. 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. Notably, in anisotropy measurements carried out with DPH we observed different trends: quercetin 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 quercetin.

4.5 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 μM verapamil, and 50-150 μM concentration of different polyphenols. 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. On the other hand, we observed additive effect of the verapamil-quercetin combination in daunorubicin accumulation assay. Verapamil and naringenin showed additive effects both in calcein and daunorubicin accumulation experiments. The combination of verapamil and ellagic acid additively increased the intracellular accumulation of calcein. In accordance with the strong ABCB1 inhibitory effect of the combined treatments, we observed elevated UIC2 reactivity compared to the only-verapamil treated samples.

Interestingly, the ellagic acid-verapamil combination decreased the daunorubicin accumulation compared to the only-verapamil treated sample. Since ellagic acid also decreased the daunorubicin staining of ABCB1-negative control cells, it is likely that ABCB1-independent mechanisms are also involved. 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, and thus it may prevent the binding and sequestration of other DNA-specific drugs, such as daunorubicin.

SECTION II

4.6 COMBINED EFFECT OF QUERCETIN AND CYANIDIN-3O-SOPHOROSIDE ON THE ATPASE AND TRANSPORT ACTIVITIES OF ABCB1

In the previous experiments, we have found that QUR hampers both the transport and ATPase activity of ABCB1 supporting that it is an ABCB1 inhibitor. On the other hand, C3S stimulates the ATPase activity and causes only a weak inhibition of substrate transport indicating that it is a substrate of ABCB1. 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.

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 μM concentration brought about a strong transport inhibitory effect, which was greater than the sum of the individual effects of each compound. The observed inhibitory effects are 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.

4.7 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. When added individually, the two polyphenols hardly affected the UIC2 reactivity of the ABCB1-positive NIH 3T3 MDR1 cells. However, an almost three-fold increase of the UIC2 reactivity was detected when the two compounds were applied simultaneously at 100 μM concentration, in accordance with the strong ABCB1-inhibitory effect of the C3S-QUR combination.

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 large 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.

4.8 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 Å). 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. 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.

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.

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.

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 account of the flexibility of side-chain residues and the protein backbone, which cannot be fully captured in docking studies. Therefore, we conducted MD simulations by the help of expert to gain further insights into the ligand-ABCB1 interactions in the above-described ligand-transporter complexes.

4.9 ANALYSIS OF HYDROGEN BONDING BETWEEN POLYPHENOLS AND AMINO ACID RESIDUES 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. Therefore, we analyzed the hydrogen bond interactions in the three polyphenol-ABCB1 complexes using MD simulations. However, upon MD simulations some of the hydrogen bonding interactions seemed dynamic by braking and reforming, while others turned out to be non-stabile and thus, they completely disappeared and new bonds were formed. Following the formation and braking of hydrogen bonds during the entire length of MD simulations, we selected the hydrogen bonding interactions with highest occupancy and stability. 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. 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, tariquidar, elacridar, benzophenone sulphonamide derivatives, CsA, carvedilol and doxorubicin.

4.10 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.

The MM-PBSA results for the 2×C3S-ABCB1 complex showed that C3S at site 1 has somewhat lower binding energy (ΔG_{bind} =-95.413 kJ/mol) compared to C3S at site 2 (ΔGbind =-87.369 kJ/mol). However, when both ligands were considered together the resulting collective binding free energy (ΔG_{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 C3S at the same site. 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.

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.

5. 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 favorable 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 favorable 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.

6. LIST OF PUBLICATIONS

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Subject: 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
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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.
   DOI: http://dx.doi.org/10.3390/pharmaceutics13122062
   IF: 6.525

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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DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

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