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Effects of chrysin and its major conjugated metabolites chrysin-7-sulfate and chrysin-7-glucuronide on cytochrome P450 enzymes, and on OATP, P-gp, BCRP and MRP2 transporters

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Running title: Pharmacokinetic interactions of chrysin and its conjugates

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Non-standard abbreviations: ABC, ATP Binding Cassette; BCRP, breast cancer resistance protein; BSP, bromosulfophthalein, C7G, chrysin-7-glucuronide; C7S, chrysin-7-sulfate; CDCF, 5(6)-Carboxy-2′,7′-dichlorofluorescein; CHR, chrysin; CYP, cytochrome P450; KET, ketoconazole; LY, lucifer yellow; MRP2, multidrug resistance-associated protein 2; NMQ, N-methyl-quinidine; OATP, Organic anion-transporting polypeptide; P-gp, P-glycoprotein; PRO, probenecid; QUI, quinidine; RMSD, root mean squared deviation; SULF, sulfaphenazole; SULT, sulfotransferase; TIC, ticlopidine; UGT, uridine 5'-diphospho-glucuronosyltransferase; VAN, vanadate; VER, verapamil

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Abstract

Chrysin is an abundant flavonoid in nature, and it is also contained by several dietary supplements. Chrysin is highly biotransformed in the body, during which conjugated metabolites, chrysin-7-sulfate and chrysin-7-glucuronide are formed. These conjugates appear at considerably higher concentrations in the circulation than the parent compound. Based on previous studies, chrysin can interact with biotransformation enzymes and transporters; however, the interactions of its metabolites have been barely examined. In this *in vitro* study, the effects of chrysin, chrysin-7-sulfate, and chrysin-7-glucuronide on CYP (2C9, 2C19, 3A4, and 2D6) as well as on Organic anion-transporting polypeptides (OATPs, 1A2, 1B1, 1B3, and 2B1) and ATP Binding Cassette (ABC: P-gp, MRP2, and BCRP) transporters were investigated. Our observations revealed that chrysin conjugates are strong inhibitors of certain biotransformation enzymes (e.g., CYP2C9) and transporters (e.g., OATP1B1, OATP1B3, OATP2B1, and BCRP) examined. Therefore, the simultaneous administration of chrysin-containing dietary supplements with medications needs to be carefully considered due to the possible development of pharmacokinetic interactions.

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Significance statement

Chrysin-7-sulfate and chrysin-7-glucuronide are the major metabolites of flavonoid chrysin. In this study, we examined the effects of chrysin and its conjugates on cytochrome P450 enzymes, and on Organic anion-transporting polypeptides and ATP Binding Cassette transporters (P-gp, BCRP, and MRP2). Our results demonstrate that chrysin and/or its conjugates can significantly inhibit some of these proteins. Since chrysin is also contained by dietary supplements, high intake of chrysin may interrupt the transport and/or the biotransformation of drugs.

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1. Introduction

Flavonoids are natural phenolic compounds in many edible plants, exerting some beneficial health effects (Kumar and Pandey, 2013). Flavonoids are rapidly biotransformed in the body, typically their metabolites reach high concentrations in the systemic circulation (and likely in some tissues) (Manach et al., 2005). As previous studies demonstrated, flavonoids can interfere with the pharmacokinetics of simultaneously administered drugs, due to the interaction with biotransformation enzymes and drug transporters (Miron et al., 2017; Vida et al., 2019). Chrysin (5,7-dihydroxyflavone; Figure 1) is a flavone aglycone which appears in some plants, honey, and propolis (Siess et al., 1996). Despite the absence of clear human evidence, chrysin-containing dietary supplements are advertised to treat depression, anxiety, hypertension, and even cancer (Mohos et al., 2018a). Furthermore, chrysin is used to maintain testosterone levels, since it inhibits the aromatase enzyme (Kao et al., 1998). Many dietary supplements contain chrysin as their active ingredient. Some of the marketed tablets/capsules include 500 mg or even higher amounts of the aglycone, and the recommended daily dose can be even 1-2 g based on the description of the manufacturers (Mohos et al., 2018a).

Chrysin has low oral bioavailability because of its significant presystemic metabolism to chrysin-7-sulfate (C7S) and chrysin-7-glucuronide (C7G) in enterocytes and hepatocytes by sulfotransferase (SULT) and uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes, respectively (Walle et al., 2001; Galijatovic et al., 1999; Ge et al., 2015). Only limited data are available regarding the pharmacokinetics of chrysin in humans. Walle et al. (Walle et al., 2001) reported that after the oral administration of a 400-mg single dose of chrysin to healthy human subjects, peak plasma concentration of the parent compound was only 16-63 nmol/L, while C7S appeared approximately at 400-800 nmol/L concentrations in the circulation. In rodent experiments, peak plasma concentrations of C7S and C7G were also considerably higher than that of chrysin (Ge et al., 2015; Dong et al., 2016; Noh et al., 2016). In mice, the

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20 mg/kg oral dose of chrysin caused 10, 130, and 160 nM peak plasma concentrations of chrysin, C7S and C7G, respectively (Ge et al., 2015). After the oral administration of 30 mg/kg and 100 mg/kg chrysin to rats, C_{max} values of C7G were approximately 750 nM (Dong et al., 2016) and 850 nM (Noh et al., 2016), respectively. In the latter study, micromolar concentrations of C7G also appeared in some rodents. Animal studies suggest the potential involvement of MRP2 and BCRP transporters in the disposition (e.g., biliary excretion) of chrysin and/or its conjugates (Walle et al., 2001; Ge et al., 2015), and the pharmacokinetic interactions of chrysin with some compounds due to the inhibition of CYP enzymes or BCRP were also reported (Wang and Morris, 2007; Kawase et al., 2009; Noh et al., 2016; Pingili et al., 2019).

The cytochrome P450 (CYP) enzyme superfamily plays an important role in the biotransformation of drugs and xenobiotics (McDonnell and Dang, 2013). Based on *in vitro* studies, chrysin is a potent inhibitor of some CYP enzymes, including CYP2C9 and 3A4 (Tsujimoto et al., 2009; Kimura et al., 2010; Ho and Saville, 2001; Noh et al., 2016). Since CYP-catalyzed oxidation is the major elimination route of several drugs, inhibitors of CYP enzymes can interfere with the pharmacokinetics of medications.

Organic anion-transporting polypeptides (OATPs) are membrane transporters that mediate the uptake of large organic anions and amphipatic molecules, including steroid and thyroid hormones, bile acids, and bilirubin. Among these polyspecific OATPs, OATP1B1 and OATP1B3 are exclusively expressed in hepatocytes (König et al., 2000), and are considered as key determinants in the hepatic uptake of numerous drugs (Shitara et al., 2013; Durmus et al., 2016). The ubiquitously expressed OATP1A2 and OATP2B1 are also polyspecific transporters. They influence hepatic (OATP2B1), intestinal (OATP1A2 and OATP2B1), and central nervous system uptake (OATP1A2 and OATP2B1) of their substrates (Shitara et al., 2013; Nakanishi et al., 2012; Urquhart and Kim 2009). OATPs commonly play a key role in

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the absorption, tissue distribution, and elimination of drugs. Therefore, their inhibition may result in the altered pharmacokinetics of simultaneously administered medications.

Human ABC (ATP Binding Cassette) drug transporters, expressed in important tissue barriers, also play a significant role in the elimination and tissue distribution of drugs, toxic compounds, or metabolites. P-gp/ABCB1, BCRP/ABCG2, and MRP2/ABCC2 multidrug transporters form together an effective defense system in the canalicular surface of hepatocytes (Szakács et al., 2008; Sarkadi et al., 2006; Jetter et al., 2020; Marquez and Van Bambeke, 2011; Wlcek and Stieger, 2014). Previous publications revealed that flavonoids, including chrysin, are inhibitors of certain ABC transporters; and some flavonoids are also substrates of these transporters (Alvarez et al., 2010; Schumacher et al., 2010; Zhang et al., 2004; Walle et al., 1999; Morris and Zhang, 2006; Tran et al., 2011). Flavonoid conjugates can be transported by ABC transporters as well; as it was concluded in a few particular reports for BCRP and MRP2 (Ge et al., 2015; Walle et al., 1999; Li et al., 2015).

In this study, the interactions of chrysin, and its major metabolites (C7S and C7G) were investigated with CYP (2C9, 2C19, 3A4, and 2D6) enzymes and with drug transporters (OATPs, P-gp, MRP2, and BCRP) employing *in vitro* assays. Our results demonstrate that both the aglycone and its conjugates are potent inhibitors of some CYP enzymes and drug transporters tested, which underlines the potential pharmacokinetic interactions of chrysin with drugs or endogenous substrates.

2. Materials and Methods

2.1. Reagents

Chrysin, testosterone, 6β-hydroxytestosterone, ketoconazole, sulfaphenazole, ticlopidine hydrochloride, quinidine, CypExpress™ 2C9, 2C19, 3A4 and 2D6 human kits, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, pyranine (trisodium 8-hydroxypyrene-

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1,3,6-trisulfonate), bromosulfophthalein, sulforhodamine 101, sodium orthovanadate, probenecid, verapamil, 5(6)-Carboxy-2′,7′-dichlorofluorescein (CDCF), lucifer yellow (LY), and benzbromarone were purchased from Sigma-Aldrich (St. Louis, MO, US). Chrysin-7-glucuronide (C7G), diclofenac, 4'-hydroxydiclofenac, S-mephenytoin, 4-hydroxymephenytoin, dextromethorphan, and dextrorphan were obtained from Carbosynth (Berkshire, UK). N-methyl-quinidine (NMQ) and Ko143 were purchased from Solvo Biotechnology (Budaörs, Hungary) and Tocris Bioscience (Bristol, UK), respectively. Chrysin-7-sulfate (C7S) was synthetized as described (Mohos et al., 2018a; Huang et al., 2006).

2.2. CYP assays

Inhibition of CYP enzymes were tested *in vitro*, using CypExpress™ Cytochrome P450 human kits. The FDA-recommended substrates (CYP2C9: diclofenac, CYP2C19: S-mephenytoin, CYP3A4: testosterone, CYP2D6: dextromethorphan) and positive controls (CYP2C9: sulfaphenazole, CYP2C19: ticlopidine, CYP3A4: ketoconazole, CYP2D6: quinidine) were applied. Chrysin, C7S, and C7G were dissolved in DMSO (that did not exceed 0.6 % in samples), solvent controls were also applied.

Inhibitory effects of chrysin, C7S, and C7G on CYP2C9 (Mohos et al., 2020), CYP2C19 (Fliszár-Nyúl et al., 2019), and CYP3A4 (Mohos et al., 2018b) enzymes were investigated as it has been reported, without modifications. Inhibition of CYP2D6 enzyme by chrysin and its conjugates was examined based on their effects on CYP2D6-catalyzed *O*-demethylation of dextromethorphan. Incubations were performed with 5 μM dextromethorphan and 10 mg/mL CypExpress™ 2D6 kit (containing glucose-6-phosphate dehydrogenase), nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$, 200 μM), and glucose-6-phosphate barium salt (G6P, 500 μM) in the presence of chrysin, C7S, or C7G (0-30 μM) in 0.05 M

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potassium phosphate buffer (pH 7.5; final volume of incubates: 200 µL). Assays were started with the addition of the enzyme and stopped with 100 µL ice-cold methanol. Samples were incubated in a thermomixer (20 min, 600 rpm, 30°C). After centrifugation (10 min, 14,000 g, room temperature), the concentrations of dextromethorphan and dextrorphan were directly analyzed from the supernatants with HPLC (see in 2.3). To determine IC_{50} values, metabolite formation (% of control) was plotted as the function of the concentrations of inhibitors in a decimal logarithmic scale, then data were evaluated using GraphPad Prism 8 software (San Diego, CA, USA).

2.3. HPLC analyses

Substrates and products of CYP enzymes were separated and quantified employing the HPLC system built up by a Waters 510 pump (Milford, MA, USA), a Rheodyne 7125 injector (Berkeley, CA, USA) with a 20-µL sample loop, and a Waters 486 UV detector. Chromatograms were evaluated applying Waters Millennium Chromatography Manager.

To quantify substrates and products in CYP2C9, 2C19, and 3A4 assays, the previously described HPLC methods were applied, without modifications (Fliszár-Nyúl et al., 2019; Mohos et al., 2018b). Regarding CYP2D6 assay, dextromethorphan and dextrorphan were quantified employing a Phenomenex Security Guard (C8, 4.0 × 3.0 mm; Torrance, CA, USA) guard cartridge linked to a Teknokroma Mediterranea Sea8 (C8, 150 × 4.6 mm, 5 µm; Barcelona, Spain) analytical column. The mobile phase contained 6.9 mM sodium acetate buffer (pH 4.0) and acetonitrile (69:31 v/v%). The isocratic elution was performed at 1.0 mL/min flow rate at room temperature, dextromethorphan and dextrorphan were detected at 280 nm. This HPLC method was suitable for the separation and quantification of the substrate and the product in the presence of chrysin and C7S; however, C7G co-eluted with dextrorphan. Therefore, the analyses of incubates with C7G was carried out with a modified

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eluent, containing 6.9 mM sodium acetate buffer (pH 4.0) and acetonitrile (72:28 v/v%). Other chromatographic parameters remained unchanged.

2.4. Modeling studies

Ligand structures of chrysin and C7S were built in Maestro (Schrödinger, 2019) and energy-minimized with a quantum chemistry program package, MOPAC (Stewart, 1990) with a PM7 parametrization (Stewart, 2013) and a gradient norm set to 0.001. Force calculations were also performed using MOPAC, the force constant matrices were positive definite. Gasteiger-Marsilli partial charges were assigned in AutoDock Tools (Morris et al., 2009). Flexibility was allowed on the ligand at all active torsions. These prepared structures were used for docking.

Atomic coordinates of the target structures were obtained from the Protein Data Bank (PDB, retrieved 18.11.2019). For CYP2C9 only one apo structure (PDB code 1og2) was available and used as a target. Holo structure for CYP2C9 (PDB code: 1og5) was applied to extract the binding mode of *S*-warfarin for comparison as a reference. For CYP2C19, the only available structure was a cytochrome target (PDB code: 4gqs) in complex with (4-hydroxy-3,5-dimethylphenyl)(2-methyl-1-benzofuran-3-yl)methanone (CYP2C19 reference ligand). Atomic partial charges of heme were adopted as the ferric penta coordinate high spin charge model from reference (Shahrokh et al., 2012). The rest of the target molecules were equipped with polar hydrogen atoms and Gasteiger-Marsilli partial charges in AutoDock Tools.

Ligand structures were docked to the active site of the targets using AutoDock 4.2.6 (Morris et al., 2009). The number of grid points was set to 90×90×90 at a 0.375 Å grid spacing. Lamarckian genetic algorithm was used for global search, with the flexibility of all active torsions allowed on the ligand (Hetényi and van der Spoel, 2006). Ten docking runs were performed, and the resulted ligand conformations were ranked according to their calculated

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binding free energy values. Representative docked ligand conformations were used for subsequent evaluations and collection of the interacting target amino acid residues with a 3.500 Å cut-off distance calculated for heavy atoms. Root mean squared deviation (RMSD) values were calculated between the crystallographic and representative ligand conformations, if available.

2.5. OATP overexpressing cell lines and OATP interaction tests

A431 cells overexpressing human OATPs, 1A2, 1B1, 1B3 or 2B1, or their mock transfected controls were generated as previously described (Patik et al., 2018; Bakos et al., 2019). Briefly, overexpression of OATP2B1 was achieved by transposase-mediated genetic insertion of the OATP2B1 cDNA (BC041095.1, HsCD00378878), followed by puromycin selection (1 μg/mL for 2 weeks). Regarding OATPs 1A2 (BC042452, HsCD00333163), 1B1 (Gene ID: AB026257), and 1B3 (BC141525, HsCD00348132), overexpression was achieved by lentiviral transduction. In order to achieve maximal OATP expression, cells were further sorted based on Live/Dead Green uptake (Patik et al., 2018; Bakos et al., 2019). OATP expression was continuously monitored by functional measurements. Cells were used for maximum 10 passages until which protein expression was stable. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fischer Scientific, Waltham, MA, US) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin, at 37^{o}C with 5% CO_2.

The interaction between chrysin and its conjugates was investigated in an indirect assay (Patik et al., 2018) employing two recently described fluorescent dye substrates, pyranine and sulforhodamine 101 (Bakos et al., 2019; Székely et al., accepted manuscript) as an indicator of OATPs' function. Briefly, A431 cell overexpressing OATPs, 1A2, 1B1, 1B3 or 2B1, or their mock transfected controls (Patik et al., 2018) were seeded on 96-well plates in a density

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of $8x10^4$ cells /well in 200 µL DMEM one day prior to the transport measurements. Next day, the medium was removed and the cells were washed three times with 200 µL phosphate-buffered saline (PBS, pH 7.4) and pre-incubated with 50 µL uptake buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 12 mM $MgSO_4$, 25 mM MES (2-(N-morpholino)ethanesulfonic acid, and 5.6 mM glucose, pH 5.5) with or without increasing concentrations of bromosulfophthalein (as a reference inhibitor), chrysin, C7S, or C7G at 37°C. Each test compound was dissolved in DMSO (that did not exceed 0.5% in samples), solvent controls were also applied. The reaction was started by the addition of 50 µL uptake buffer containing pyranine in a final concentration of 10 µM (OATP1B1) or 20 µM (OATP1B3 and OATP2B1), or 0.5 µM sulforhodamine 101 (OATP1A2). Thereafter, cells were incubated at 37°C for 15 min (OATP1B1 and OATP2B1), 10 min (OATP1A2), or 30 min (OATP1B3). The reactions were stopped by removing the supernatants, then the cells were washed three times with ice-cold PBS. Fluorescence (in 200 µL PBS/well) was determined employing an Enspire plate reader (Perkin Elmer, Waltham, MA) ex/em: 403/517 nm (pyranine) or 586/605 nm (sulforhodamine 101). OATP-dependent transport was calculated by extracting fluorescence measured in mock transfected cells. Transport activity was calculated based on the fluorescence signal in the absence (100%) of chrysin or its conjugates. Experiments were repeated in three biological replicates. IC_{50} values were calculated by Hill1 fit, using the Origin Pro8.6 software (GraphPad, La Jolla, CA, USA).

2.6. Transport activity measurements for ABC transporters

Transport activity was measured in insect membrane vesicles, as it has been reported earlier (Sarkadi et al., 1992; Bodo et al., 2003; Ozvegy et al., 2001; Ozvegy et al., 2002; Telbisz et al., 2007). Briefly, human P-gp, BCRP, and MRP2 were expressed in Sf9 insect cells by baculoviruses. At the third day of infection, cells were collected and membrane vesicles were

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obtained by mechanical disruption and differential centrifugation. For BCRP, the cholesterol level of the vesicles was adjusted to the level of mammalian membranes to get full activity (Telbisz et al., 2007). Membrane vesicles were stored at -80^{o}C and total protein content of preparations was measured by Lowry method and used as a reference of the quantity. Appropriate amount of membrane vesicles (50 μg protein/sample) was incubated with transporter specific fluorescent substrates (BCRP: 10 μM lucifer yellow (LY), ABCB1: 5 μM n-methyl-quinidine (NMQ), MRP2: 5 μM 5(6)-Carboxy-2′,7′-dichlorofluorescein (CDCF)) at 37^{o}C for 10 min without or with 4 mM of Mg-ATP in 50 μL volume. Quality of membrane vesicles was confirmed applying known reference inhibitors (see details in the Results section). ATP dependent uptake of fluorescent compounds was investigated in the presence of flavonoids (up to 50-200 μM). Each test compound was dissolved in DMSO. Although DMSO (2 v/v% final concentration in each sample) did not affect the assays used, the solvent controls were represented in the corresponding figures. After incubation, samples were rapidly filtered and washed on filter plate (MSFBN6B10, Millipore, Burlington, MA, US). Accumulated substrates in vesicles were solved back from the filter by 100 μL of 10% SDS and centrifuged into another plate. A 100 μL volume of fluorescence stabilizer additive was added to the samples (DMSO for LY, 0.1 M H_2SO_4 for NMQ, and 0.1 M NaOH for CDCF). Fluorescence of samples were measured by plate readers (Victor X3 and Enspire Perkin-Elmer, Waltham, MA, US) at appropriate wavelengths (filters in Victor X3 reader were 405 nm and 535 nm for LY, 492 nm and 635 nm for CDCF; and in Enspire reader NMQ was measured at 360 and 430 nm excitation and emission wavelengths, respectively). ABC related transport was calculated by subtracting passive uptake measured without Mg-ATP from values measured in the presence of Mg-ATP. The backgrounds with and without Mg-AMP were the same. Under the applied concentrations, we did not observe considerable quenching effects of flavonoids.

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2.7. ATPase activity assays for ABC transporter interactions

ATPase activity was measured on Sf9 membrane vesicles containing human ABC transporters prepared as described in 2.6. Appropriate amount of vesicles (BCRP: 10 μg/50 μL, P-gp: 20 μg/50 μL, MRP2: 30 μg/150 μL) was used in assays. Other parameters have been described previously (Sarkadi et al., 1992; Bodo et al., 2003; Ozvegy et al., 2001; Ozvegy et al., 2002; Telbisz et al., 2007). Membrane vesicles were incubated with 3 mM of Mg-ATP for 25 (BCRP and P-gp) or 60 min (MRP2) at 37^{o}C. Effects of flavonoids were investigated up to 50-200 μM. Chrysin, C7S, and C7G were dissolved in DMSO. Although DMSO (2 v/v% final concentration in each sample) did not affect the assays used, the solvent controls were represented in the corresponding figures. ABC transporter related activity was determined as vanadate sensitive ATPase activity. Liberated inorganic phosphate was measured by a colorimetric reaction, as described (Sarkadi et al., 1992). Absorbance of samples were measured after 25 min at 660 nm.

2.8. Statistics

The statistical significance ($p < 0.05$ and $p < 0.01$) was established based on one-way ANOVA test, using Tukey's post-hoc test (IBM SPSS Statistics, Armonk, NY, US).

3. Results

3.1. Inhibition of CYP enzymes by chrysin, C7S, and C7G

The effects of chrysin, C7S, C7G, and the positive control sulfaphenazole on CYP2C9 are demonstrated in Figure 2A. C7G did not inhibit the enzyme, even at 30 μM concentration. In contrast, chrysin (IC_{50} = 3.2 μM) and C7S (IC_{50} = 2.7 μM) proved to be strong inhibitors of

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CYP2C9, showing only 2.5- and 2-fold weaker effects vs. sulfaphenazole (IC_{50} = 1.3 μM), respectively.

The impacts of flavonoids and the positive control ticlopidine on the CYP2C19 are demonstrated in Figure 2B. C7S and C7G exerted statistically significant but only slight inhibitory effects on the enzyme. However, chrysin (IC_{50} = 4.6 μM) caused similarly potent inhibition on CYP2C19 to ticlopidine (IC_{50} = 4.4 μM).

The influence of chrysin and its conjugated metabolites on CYP3A4 is demonstrated in Figure 2C. C7G did not affect the enzyme, while chrysin and C7S induced statistically significant but only slight inhibition of CYP3A4. Furthermore, under the applied conditions, chrysin and its conjugates did not inhibit CYP2D6 (Figure 2D).

3.2. Modeling Studies

The ligand binding of *S*-warfarin (reference ligand for CYP2C9) was reproduced at an RMSD value of 1.240 Å in the top 1st rank (PDB structure: 1og5). Re-docking of the reference ligand of CYP2C19 resulted in a somewhat increased RMSD value of 6.590 Å as top 1st rank still located at the experimental binding position. New ligands of the present study (chrysin and C7S) were also docked to the respective binding pockets of the enzymes located above the heme ring. Regarding the binding mode to CYP2C9, chrysin found a binding position which appears to be final (due to the close coordination of the benzene ring to the heme iron) with closest distances of 3.700 Å measured between benzene C atoms and heme Fe^{3+}, respectively (Figure 3A and 3B). This final position was probably acquired through a couple of prerequisite binding positions. Chrysin reached the final position in the top 5th rank. C7S remained at a prerequisite position of the previous ligands (Figure 3A and 3C), without moving close to the heme iron. The calculated binding free energies suggest that C7S (-7.78 kcal/mol) binds with a higher binding affinity to CYP2C9 than chrysin (-6.11 kcal/mol),

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which is in agreement with our experimental observations. Regarding CYP2C19, the binding free energy of chrysin was -6.36 kcal/mol. Chrysin found its final binding position through one prerequisite step in top 2nd rank position. The closest distances between the heme Fe^{3+} and benzene C atoms of chrysin was 3.600 Å (Figure 3D and 3E).

3.3. Inhibition of OATP activity by chrysin, C7S, and C7G

Fluorescent substrates, pyranine and sulforhodamine 101 have recently been demonstrated as good indicators of substrate/inhibitor interactions of OATPs (Bakos et al., 2019; Székely et al., accepted manuscript). Uptake of these dyes was determined in A431 cells, engineered to overexpress one of the multispecific OATPs (1A2, 1B1, 1B3, or 2B1), and bromosulfophthalein was used as a reference inhibitor (Patik et al., 2018; Bakos et al., 2019). Figure 4A represents that chrysin is not a potent inhibitor of OATP1A2: only 40% inhibition was achieved in the presence of 100 μM chrysin concentration. In contrast, C7S and C7G showed stronger interactions with OATP1A2 (IC_{50} = 18.3 and 24.1 μM, respectively). Regarding OATP1B1 and OATP1B3, chrysin proved to be again a weak inhibitor (IC_{50} > 100 μM) (Figures 4B and 4C). However, C7S and C7G revealed more pronounced inhibition for both proteins than chrysin, C7S being a quite effective inhibitor of OATP1B1 (IC_{50} = 0.8 μM) and OATP1B3 (IC_{50} = 1.7 μM). C7G also showed relatively low IC_{50} values (OATP1B1: 4.4 μM; OATP1B3: 14.3 μM). In addition, among the four OATPs tested, OATP2B1 was the most sensitive to chrysin, C7S, and C7G, showing 4.8, 0.5, and 0.3 μM IC_{50} values, respectively (Figure 4D).

3.4. Effects of chrysin, C7S, and C7G on ABC transporters

For the characterization of the transport activity of BCRP, ATP dependent uptake of LY was measured. Ko143 was used as a reference inhibitor, it decreased ATP dependent LY uptake

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by more than 95% at 5 μM concentration (data not shown). Chrysin and its sulfate conjugate strongly decreased LY uptake (Figure 5A). IC_{50} values of chrysin and C7S were around 0.4-0.6 μM (and at 10 μM total inhibition was observed for both), while C7G caused much weaker inhibition (IC_{50} = 19.8 μM). The vanadate sensitive ATPase activity in the presence of flavonoids was also tested. Each compound increased basal ATPase activity in a concentration dependent fashion (Figure 5B). Consistent with the IC_{50} values, chrysin and C7S showed a lower K_m (0.1 μM) and higher V_{max} (90 and 70 nmol Pi/min/mg protein, respectively) values than C7G (K_m = 8.4 μM).

MRP2 typically has low substrate affinity and a low ATPase activity (Bodo et al., 2003), therefore, compounds were tested at higher concentrations. MRP2 transport activity was measured by ATP dependent CDCF transport which was sensitive to benzbromarone and partially to vanadate (data not shown). MRP2-mediated (ATP dependent) uptake of CDCF was considerably decreased by C7G (IC_{50} = 11.2 μM), whereas chrysin and C7S had no and minor effects, respectively (Figure 5C). Since we observed a faint ATP dependent CDCF transport in mock membranes as well, we demonstrated the measurements with chrysin and its conjugates with mock membranes (Fig 5C). Accordingly, only in the presence of C7G can be an increased but faint (it did not exceed 6-7 nmol Pi/min/mg protein) ATPase activity observed (Figure 5D). In these measurements, probenecid was also applied as a positive control (Bakos et al., 2000; Huisman et al., 2005).

Despite the fact that P-gp shares several substrates with BCRP, we found that P-gp mediated NMQ uptake (which was completely inhibited by 1 mM vanadate or 30 μM verapamil) was not inhibited by chrysin, C7S, and C7G. However, chrysin significantly stimulated the transport (Figure 5E). To confirm the P-gp related phenomenon, NMQ uptake in mock membrane vesicles was also tested. In control vesicles, NMQ uptake was not influenced by the presence of flavonoids (Figure 5E). In the ATPase assay, we did not find any significant

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effect of chrysin and its conjugates even at 50 μM concentrations (Figure 5F); however, the positive control verapamil strongly increased the basal ATPase activity.

4. Discussion

Since high dose flavonoid-containing dietary supplements are widely advertised and marketed on the Internet for various purported uses (Vida et al., 2019), there is a high probability that some people apply these supplements together with their prescribed medicines. Because the pharmacokinetic interactions of dietary supplements are poorly characterized, in this study, the interactions of chrysin and its main conjugates with CYP enzymes, OATP uptake transporters, and ABC exporters were examined.

Chrysin showed potent inhibitory effects on CYP2C9 and 2C19. In other *in vitro* studies performed with diclofenac (IC_{50} = 6.5 μM) and flurbiprofen (IC_{50} = 0.8 μM) substrates, chrysin also strongly inhibited CYP2C9 at comparable concentrations with the substrates used (Kimura et al., 2010; Shimada et al., 2010). Furthermore, propolis extract (containing 23.6 μg/mg chrysin) significantly inhibited CYP2C19 (Ryu et al., 2016). Previous investigations with testosterone (IC_{50} = 0.9 and 7.4 μM) (Tsujimoto et al., 2009; Kimura et al., 2010), midazolam (IC_{50} = 3.8 μM) (Shimada et al., 2010), and quinine (IC_{50} = 70-86 μM) (Ho and Saville, 2001) substrates suggest the interaction of chrysin with CYP3A4. However, we found only a weak inhibitory effect of chrysin on CYP3A4. In a recent study, chrysin did not inhibit CYP2D6 (substrate: dextromethorphan) (Bojić et al., 2019), which is in agreement with our results. We did not find any data in the scientific literature regarding the inhibitory effects of C7S and C7G on CYP enzymes. In this study, C7G showed no or negligible inhibitory effects on CYP enzymes tested, while C7S proved to be even a stronger inhibitor of CYP2C9 than chrysin (but it had only weak effects on CYP2C19 and 3A4). Based on these observations, we

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may expect the slower elimination of CYP2C9 substrates as a result of the high consumption of chrysin.

We identified the key interacting residues involved in the binding of chrysin and C7S to CYP2C9 and/or 2C19. Chaichit et al. (2014) investigated the binding of chrysin to CYP2C9, they also identified F114, L208, and A297 as key interacting amino acids. A previous *in silico* study of flavonoid-CYP2C9 interactions (Sousa et al., 2013) suggests the importance of N474 and S209, we have the same findings regarding C7S (Fig. 3C). The binding free energies of naringenin (-5.98 kcal/mol) and quercetin (-6.77 kcal/mol) with CYP2C9 (Sousa et al., 2013) showed good correlation with our data. Furthermore, previous *in silico* studies with bavachin also suggest that the binding position of flavonoids is close to the heme iron in both CYP2C9 and 2C19 (Wang et al., 2018), as it is demonstrated with chrysin in Fig. 3A and D. Investigation of corylifol A showed the importance of amino acid residues L208, A297, and T301 in the interaction with CYP2C9 enzyme, and the involvement of F100, F114, N204, L237, G296, L366, and F476 has been reported with CYP2C19 (Wang et al., 2018), showing good correlations with our observations.

The interaction between flavonoids and OATPs has been reviewed in several reports (Miron et al., 2017; Čvorović et al., 2018). Based on previous studies, chrysin seems to be a weak inhibitor of OATP1B1 (33% inhibition of estrone-3-sulfate uptake was observed by 100 μM chrysin) (Wu et al., 2012; Wang et al., 2005), and a potent inhibitor of OATP2B1 and OATP1A2 (Navrátilová et al., 2018). However, the interaction of chrysin metabolites with OATPs has not yet been investigated. We demonstrated here that chrysin is a potent inhibitor of OATP2B1 (IC_{50} = 4.8 μM), which is in agreement with the inhibition of OATP2B1-mediated estrone-3-sulfate uptake by chrysin (observed in MDCKII cells) (Navrátilová et al., 2018). Since OATP2B1 is expressed in enterocytes (Kobayashi et al., 2003), this transporter may be involved in the absorption of chrysin. The indirect transport assay applied in the

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current study does not distinguish competitive (substrate) and non-competitive inhibitors. Walle et al. reported a well-measurable apical-to-basolateral transport of chrysin (Walle et al., 1999); however Rastogi and Jana suggested passive diffusion as the main route of chrysin absorption (Rastogi et al., 2016). Therefore, further investigations are reasonable to make this issue clear. Based on our data, no significant interaction can be expected between chrysin and the other multispecific OATPs, 1A2, 1B1, and 1B3. In accordance with our observations, a weak or negligible inhibitory effect of chrysin on OATP1B1 was reported (Wu et al., 2012; Wang et al., 2005). On the other hand, the potent inhibition (IC_{50} = 0.15 μM) of OATP1A2-mediated estrone-3-sulfate uptake was noticed in HEK 293 cells (Navrátilová et al., 2018). This discrepancy may be explained by the different test substrates used. In the current study, chrysin metabolites showed potent inhibitory effects on each OATP tested. C7G inhibited OATPs, 1A2, 1B1, and 1B3 in micromolar range, and attenuated OATP2B1's function with a submicromolar IC_{50} value (0.33 μM). C7S proved to be the most potent inhibitor of OATPs, with IC_{50} values of 9.8 μM (OATP1A2), 0.76 μM (OATP1B1), 1.73 μM (OATP1B3), and 0.55 μM (OATP2B1). Since *in vivo* studies suggest that the peak plasma concentrations of C7S and/or C7G can be close to 1 μM (Walle et al., 2001; Dong et al., 2016; Noh et al., 2016), they may influence the pharmacokinetics of drug or endogenous substrates of these multispecific OATPs. OATP2B1 mediates the absorption of its substrates from the gut, and (together with OATP1B1 and OATP1B3) the uptake of several compounds from the blood to hepatocytes (Kovacsics et al., 2017). OATP1A2 may have an important role in the transport of drugs through the blood-brain barrier. Considering these observations, the high intake of chrysin may interfere with the above-listed transport mechanisms. It is important to note that we used one probe substrate for each OATP, while inhibitors may have various effects depending on the substrates used (Izumi et al., 2013). Nevertheless, our data warrant further studies regarding the interactions of chrysin, C7S, and C7G with other OATP substrates.

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Importantly, OATPs are also responsible for the uptake of toxic compounds, including α-amanitin (Letschert et al., 2006). Therefore, the potent inhibition of OATPs by C7S (the major circulating metabolite of chrysin) may be beneficial due to the decreased tissue uptake of these toxins.

ABC multidrug exporters are involved in the elimination of flavonoids as extruders at important tissue barriers. Previous studies suggest that P-gp, BCRP, and MRP2 interact with chrysin either directly or by transporting outwardly its metabolites (Alvarez et al., 2010; Schumacher et al., 2010; Zhang et al., 2004; Walle et al., 1999; Morris and Zhang, 2006; Tran et al., 2011). In cells expressing these ABC exporters, chrysin inhibited BCRP in all studies but the results are controversial for P-gp (Alvarez et al., 2010; Zhang et al., 2004; Morris and Zhang, 2006; Tran et al., 2011); while the conjugates may be the substrates of BCRP and/or MRP2 exporters (Ge et al., 2015; Walle et al., 1999; Li et al., 2015; An and Morris, 2011; Wang and Morris, 2007). In the current study, inverted membranes were used, which gives the unique possibility to measure direct interactions of hydrophilic metabolites with ABC transporters. Our data show that chrysin and its metabolites are inhibitors of BCRP-mediated transport (Fig. 5A). ATPase activity measurements support the observation that chrysin and C7S have stronger inhibitory effects on BCRP compared to C7G (Fig. 5B). Since ATPase and transport activity are coupled processes and several transported flavonoids (e.g., flavopiridol, quercetin, and kaempferol) are known to increase ATPase activity of BCRP, chrysin and its conjugates may be transported by the protein. Nevertheless, these data give only circumstantial evidence; therefore, further experiments (measurement of direct transport) are reasonable to prove or reject this hypothesis. The transport activity of MRP2 was also inhibited by flavonoids tested. However, in this assay, C7G was a stronger inhibitor of MRP2 than chrysin and C7S (Fig. 5C). Flavonoids tested did not inhibit P-gp; however, chrysin significantly increased the transport of NMQ (Fig. 5E), which is in agreement with some of

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the previous studies (Tran et al., 2011; Reddy et al., 2016). Nevertheless, we cannot exclude the possibility that the latter cross-reaction is typical only for certain substrates (Tran et al., 2011; An and Morris, 2011) and resulted from special interactions between different binding sites or allosteric modifications.

Although chrysin has low oral bioavailability, its metabolites can reach high concentrations in body fluids (e.g., plasma, bile, and urine) (Walle et al., 2001; Dong et al., 2016; Noh et al., 2016). We have limited data regarding the pharmacokinetics and drug interactions of chrysin in humans; however, animal experiments showed that chrysin can influence the pharmacokinetics of different compounds (e.g., caffeine, nitrofurantoin, and paracetamol) due to its interactions with CYP enzymes and BCRP (Wang and Morris, 2007; Kawase et al., 2009; Noh et al., 2016; Pingili et al., 2019). There is only one human study where 400 mg single dose of chrysin was administered orally, after which the average C_{max} value of C7S was approximately 600 nM (Walle et al., 2001). However, the repeated administration of higher chrysin doses (even 1-2 g daily) can be estimated based on the recommended daily doses by manufacturers (Mohos et al., 2018a). Therefore, it is reasonable to hypothesize the low micromolar plasma concentrations of chrysin conjugates. In agreement with this hypothesis, the repeated administration of 1 g quercetin daily resulted in approximately 2 μM or even higher peak plasma concentration of quercetin conjugates (Conquer et al., 1998; Vida et al., 2019). Considering the above-listed data and our observations, the interactions of chrysin and/or it metabolites with CYP2C9, OATPs, and BCRP may have high pharmacological importance. Therefore, the simultaneous administration of high dose chrysin-containing dietary supplements and medications should be carefully considered, at least until clinical studies prove its safety. Since the internet sale of dietary supplements is largely uncontrolled (Vida et al., 2019), health care professionals should be aware of the risk regarding drug-supplement interactions to prevent the corresponding health impairment. Nevertheless, to

explore the *in vivo* pharmacological relevance of these interactions, animal studies need to be performed and/or human data should be analyzed and evaluated in the future.

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Wrote, or contributed to the writing of the paper: Mohos, Poór, Zsidó, Özvegy-Laczka, Telbisz

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Footnotes
(Unnumbered footnote to title)

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Figure legends

Figure 1: Chemical structures of chrysin, chrysin-7-sulfate, and chrysin-7-glucuronide (SULT, sulfotransferase; UGT, uridine 5'-diphospho-glucuronosyltransferase).

Figure 2: Concentration-dependent inhibitory effects of chrysin, C7S, C7G, and positive controls on CYP enzymes. Inhibition of CYP2C9-catalyzed 4'-hydroxydiclofenac (**A**), CYP2C19-catalyzed 4-hydroxymephenytoin (**B**), CYP3A4-catalyzed 6β-hydroxytestosterone (**C**), and CYP2D6-catalyzed dextrorphan (**D**) formation in the presence of increasing inhibitor concentrations (0-30 μM; substrate concentrations: 5 μM in each assay). Mean ±SEM values are derived from three independent experiments (CHR, chrysin; C7S, chrysin-7-sulfate; C7G, chrysin-7-glucuronide; SULF, sulfaphenazole; TIC, ticlopidine; KET, ketoconazole; QUI, quinidine; *$p < 0.05$, **$p < 0.01$).

Figure 3: (**A**) The binding modes of chrysin (space-filling, yellow) and C7S (space filling, navy blue) as docked to the binding pocket of CYP2C9 enzyme (teal cartoon) above the heme ring (green sticks). (**B**) The close-up of binding mode of chrysin (yellow sticks) as docked to the binding pocket of CYP2C9 enzyme above the heme ring (Fe^{3+} ion in orange, heme as green sticks). (**C**) The close-up of binding mode of C7S (navy blue sticks) as docked to the binding pocket of CYP2C9 enzyme. (**D**) The binding mode of chrysin (space-filling, navy blue) as docked to the binding pocket of CYP2C19 enzyme (teal cartoon) above the heme ring (green sticks). (**E**) The close-up binding mode of chrysin (navy blue sticks) as docked to the binding pocket of CYP2C19 above the heme ring (Fe^{3+} ion in orange, heme as green sticks). In panels **B**, **C**, and **E**, the interacting enzyme residues are labeled and shown as thin gray sticks.

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Figure 4: Concentration-dependent inhibitory effects of bromosulfophthalein (BSP, positive control), chrysin, C7S, and C7G on OATPs. Uptake of pyranine in A431 cell overexpressing OATPs, 1B1, 1B3 or 2B1, and the uptake of sulforhodamine 101 (OATP1A2) was measured in the presence of increasing concentrations of chrysin, C7S, or C7G (0-100 μM). Effect of BSP on CYP1A2 has been reported previously (Bakos et al., 2019). OATP-dependent transport was determined by extracting the fluorescence measured in mock transfected cells. Fluorescence with the dyes alone were set as 100%. Dye concentrations and incubation times were the following: (**A**) OATP1A2: 0.5 μM sulforhodamine 101, 10 min; (**B**) OATP1B1: 10 μM pyranine, 15 min; (**C**) OATP1B3: 20 μM pyranine, 30 min; (**D**) OATP2B1: 20 μM pyranine, 15 min. Mean ±SEM values were obtained from three biological replicates (*$p < 0.05$, **$p < 0.01$).

Figure 5: Effects of chrysin (CHR) and its metabolites on ABC transporters. ABC transporter functions were measured in human ABC transporter (BCRP, MRP2, and P-gp) containing inverted insect cell membrane vesicles. Transport activities were characterized by vesicular uptake of specific fluorescent substrates (LY for BCRP, CDCF for MRP2, and NMQ for P-gp) where transport by the ABC transporter is defined as ATP dependent uptake of the substrate into the vesicles. Vanadate sensitive ATPase activity of ABC transporters were measured by detecting inorganic, deliberated phosphate in a colorimetric reaction. (**A**) Effect of flavonoids on the transport of LY by BCRP. (**B**) Changes in the ATPase activity of BCRP in the presence of flavonoids. (**C**) Effects of flavonoids on the transport of CDCF by MRP2. Mock membrane data are also presented with dashed line. (**D**) Changes in the ATPase activity of MRP2 in the presence of flavonoids and probenecid (PRO, positive control). (**E**) Changes in the NMQ uptake into mock (white label) and P-gp (black label) containing vesicles in various conditions (VAN: vanadate; VER: verapamil). (**F**) ATPase activity of P-gp in the

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presence of various concentrations of flavonoids and verapamil (positive control). Mean ±SEM values were obtained from three biological replicates (*$p < 0.05$, **$p < 0.01$).