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Interactions of resveratrol and its metabolites (resveratrol-3-sulfate, resveratrol-3-glucuronide, and dihydroresveratrol) with serum albumin, cytochrome P450 enzymes, and OATP transporters

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ABSTRACT

Resveratrol (RES) is a widely-known natural polyphenol which is also contained by several dietary supplements. Large doses of RES can result in high micromolar levels of its sulfate and glucuronide conjugates in the circulation, due to the high presystemic metabolism of the parent polyphenol. Pharmacokinetic interactions of RES have been extensively studied, while only limited data are available regarding its metabolites. Therefore, in the current study, we examined the interactions of resveratrol-3-sulfate (R3S), resveratrol-3-glucuronide, and dihydroresveratrol (DHR; a metabolite produced by the colon microbiota) with human serum albumin (HSA), cytochrome P450 (CYP) enzymes, and organic anion transporting polypeptides (OATP) employing *in vitro* models. Our results demonstrated that R3S and R3G may play a major role in the RES-induced pharmacokinetic interactions: (1) R3S can strongly displace the site I marker warfarin from HSA; (2) R3G showed similarly strong inhibitory action on CYP3A4 to RES; (3) R3S proved to be similarly strong (OATP1B1/3) or even stronger (OATP1A2 and OATP2B1) inhibitor of OATPs tested than RES, while R3G and RES showed comparable inhibitory actions on OATP2B1.

1. Introduction

Resveratrol (RES; trans-3,4′,5-trihydroxystilbene) is a natural polyphenol which occurs *e.g.*, in grapes, peanuts, berries, and red wine [1,2]. In addition, RES also appears in certain dietary supplements, containing typically 50–2000 mg RES in one single unit (tablet/capsule), while the average dietary intake of RES ranges from the few μg/day to the few hundreds of μg/day [3,4]. RES is considered to have beneficial health effects (with limited clinical evidence): Previous studies suggest its antioxidant, anti-inflammatory, cardioprotective, neuroprotective, anticancer, and anti-aging impacts [5–8].

In humans, approximately 75% of RES is absorbed after its oral administration [9]. However, RES has a low oral bioavailability (less than 1%) due to its extensive presystemic elimination in enterocytes and hepatocytes [10]. In rodents, the major metabolites of RES are sulfate and glucuronic acid conjugates, including resveratrol-3-glucuronide (R3G) and resveratrol-3-sulfate (R3S), which can be detected in blood, liver, kidney, and urine [11,12]. Human studies also demonstrated the occurrence of sulfate and/or glucuronide conjugation of RES: Among other conjugates, R3G and R3S appeared at high concentrations in plasma and tissue samples [13–15]. The normal dietary intake of RES results in its low nanomolar concentrations in the circulation, while the administration of extremely high doses (*e.g.*, dietary supplements) can cause micromolar plasma levels [16]. Human studies demonstrated that R3G and R3S can reach approximately 10 and 20 μM peak plasma concentrations, respectively [13,17]. RES metabolites appear at considerably higher concentrations in the circulation and in some tissues compared to the parent compound [13–15], even 20-fold higher AUC

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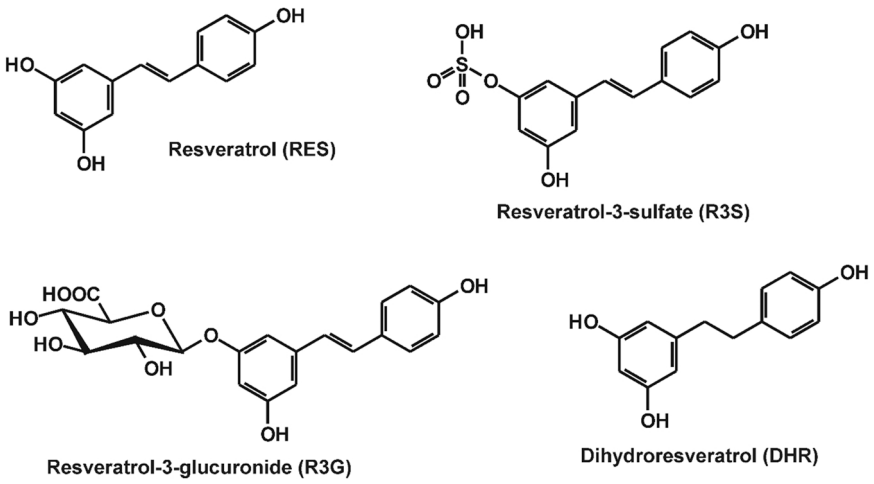

Fig. 1. Chemical structures of resveratrol (RES), resveratrol-3-sulfate (R3S), resveratrol-3-glucuronide (R3G), and dihydroresveratrol (DHR).

values of the metabolites can be noticed [17]. Besides sulfate and glucuronic acid conjugation, RES is also metabolized by the colon microflora resulting in the formation of dihydroresveratrol (DHR), which is also absorbed based on rat experiments [18].

Human serum albumin (HSA) forms stable complexes with several endogenous compounds, ions, drugs, nutrients, and toxins, affecting their pharmacokinetic/toxicokinetic properties [19,20]. The two major drug binding sites of HSA are located in subdomain IIA (Sudlow's site I) and in subdomain IIIA (Sudlow's site II) [19]. The displacement of strongly albumin-bound molecules from HSA leads to their elevated free plasma concentration, which can affect the tissue distribution of these drugs and/or the speed of their elimination [20,21]. RES binds to HSA with high affinity occupying Sudlow's site I [22–24], while the interactions of its metabolites with HSA have not yet been characterized.

Cytochrome P450 (CYP) enzymes are heme containing proteins, catalyzing primarily the oxidative biotransformation of drugs and xenobiotics [25]. Among the different CYP isoforms, CYP1A2, 2C9, 2C19, 2D6, and 3A4 are most frequently involved in the biotransformation of drugs [26]. Previous investigations suggest that RES can inhibit certain CYP enzymes, including CYP1A1, 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 [24,27–29]. In earlier *in vitro* studies, R3S showed slight or negligible inhibition on CYP1A2, 2C9, 2C19, 2D6, and 3A4 enzymes [27]; while DHR exerted only weak inhibitory impacts on CYP2C8 and 3A4/5 (but did not affect CYP1A2 and other members of the CYP2 family tested) [30]. Nevertheless, we did not find other data regarding the potential interactions of R3S, R3G, or DHR with CYP enzymes.

Organic anion transporting polypeptides (OATPs) are Solute Carrier (SLC)-type transporters expressed in the plasma membrane of human epithelial or endothelial cells [31]. OATPs typically mediate the exchange of large (>300 Da) organic anions or amphipathic molecules across the cell membranes [32]. Among the 11 human OATPs, OATP1A2, OATP1B1, OATP1B3, and OATP2B1 are multispecific transporters that besides their endogenous substrates (*e.g.*, bilirubin, steroid-hormone conjugates, or bile acids) also transport various clinically applied drugs, including statins [33,34]. OATP1B1 and OATP1B3 are (almost) exclusively expressed in hepatocytes, where they mediate the hepatocellular uptake of their substrates from the portal vein [31,35]. OATP1A2 and OATP2B1 are ubiquitously expressed in the human body, and influence the blood to brain penetration or intestinal uptake (OATP2B1) of their substrates [36,37]. Owing to their importance in pharmacokinetics, inhibition of multispecific OATPs' function by the co-administration of their substrates/inhibitors may result in altered drug plasma levels and unwanted side effects [38,39]. Therefore, and according to international (FDA, EMA) regulations, interaction between drug candidates and OATP1B1 and OATP1B3 (playing key role in the hepatic elimination of their drug substrates) have to be investigated. Not only drugs but certain nutrients (such as quercetin and other flavonoids) and/or their metabolites can also inhibit OATPs [32,40–42]. RES and its 3-*O*-sulfate or -glucuronide conjugates have also been demonstrated to inhibit OATP1B1 and OATP1B3 [43]. Moreover, 3-*O*- or 3,4-di-*O*--sulfated RES have been shown to be transported substrates of OATP1B1, OATP1B3, and/or OATP2B1 [44]. However, interaction between DHR and OATPs, 1A2, 1B1, 1B3, 2B1, or RES and its metabolites with OATP1A2 has not yet been investigated.

In this study, we aimed to examine the interactions of RES metabolites (R3S, R3G, and DHR) with HSA, CYP (2C9, 2C19, 2D6, and 3A4) enzymes, and OATP (1A2, 1B1, 1B3, and OATP2B1) transporters. Albumin-ligand interactions were evaluated based on fluorescence spectroscopy, ultrafiltration, and high-performance affinity chromatography. The inhibitory effects of RES and its metabolites on CYP enzymes were examined using *in vitro* enzyme assays, after which the substrates and metabolites were quantified by high-performance liquid chromatography (HPLC). The inhibitory effects of RES and its metabolites on OATP transport function was investigated *in vitro*, employing OATP overexpressing cells. Our results demonstrate, that not only RES but its metabolites can also interact with HSA as well as with certain CYPs and OATPs. (Fig. 1).

2. Materials and methods

2.1. Reagents

Trans-resveratrol (RES), human serum albumin (HSA), racemic warfarin, racemic naproxen, CypExpress™ Cytochrome P450 (2C9, 2C19, 2D6, and 3A4) human kits, testosterone, ticlopidine, quinidine, and ketoconazole were obtained from Merck (Darmstadt, Germany). Trans-resveratrol-3-*O*-b-D-glucuronide (R3G), diclofenac, 4′-hydroxydiclofenac, S-mephenytoin, 4-hydroxymephenytoin, dextromethorphan, dextrorphan, 6β-hydroxytestosterone, and sulfaphenazole were purchased from Carbosynth (Berkshire, UK). Trans-resveratrol-3-*O*-sulfate sodium salt (R3S) and dihydroresveratrol (DHR) were from Toronto Research Chemicals (Toronto, Canada) and Cayman Chemical Company (Ann Arbor, MI, US), respectively. Stock solutions of RES and its metabolites (each 10 mM, in dimethyl sulfoxide) were stored at − 20 °C. Fluorescent dyes and reagents for OATP assays, if not stated otherwise, were purchased from Merck (Darmstadt, Germany).

2.2. Spectroscopic studies

For the fluorescence spectroscopic investigation of albumin-ligand interactions, a Hitachi F-4500 fluorimeter (Tokyo, Japan) was applied. UV–vis spectra were recorded using a Jasco V730 UV–vis

spectrophotometer (Tokyo, Japan). Measurements were performed in phosphate-buffered saline (PBS, pH 7.4) at 25 °C. After the absorption spectra of polyphenols were recorded, the inner-filter effects were corrected in each experiment, as it has been reported [45,46].

In fluorescence quenching studies, emission spectra of HSA (2.0 μM) were collected without and with increasing polyphenol concentrations (0, 0.5, 1, 2, 3, 4, 5 and 6 μM; λ_{ex}= 295 nm). Stern-Volmer quenching constants (K_{SV}) were determined based on the changes in the emission signal at 340 nm, as it has been described previously [46,47].

Since RES, R3S, and R3G exert intrinsic fluorescence, the changes in their emission signal (each 1 μM) were tested in the presence of increasing concentrations of HSA (0, 1, 2, 3, 4, 6, 8, and 10 μM) in PBS (pH 7.4; λ_{ex} = 320 nm for each polyphenol; λ_{em} = 385 nm for RES, 385 nm and 455 nm for R3S, and 395 nm for R3G). Data were evaluated using the Benesi-Hildebrand plot, as it has been described previously [48].

2.3. High-performance affinity chromatography (HPAC)

HPAC studies [49] were performed applying the HPLC system described in Section 2.4. Samples (5 μL) were driven through a Chiralpak HSA (50 × 3.0 mm, 5 μm; West Chester, PA, US) affinity column with 0.5 mL/min flow rate. The isocratic elution was performed with sodium phosphate buffer (10 mM, pH 7.0) and isopropanol (80:20 v/v %). RES, R3S, and R3G were detected with fluorescence detector at 400 nm (λ_{ex} = 317 nm).

2.4. Ultrafiltration experiments

Effects of RES and its metabolites on the albumin binding of warfarin (site I marker) and naproxen (site II marker) were examined by ultrafiltration employing the previously reported methods [50,51]. Samples containing warfarin+HSA (1 and 5 μM, respectively) or naproxen+HSA (1 and 1.5 μM, respectively) without or with polyphenols were filtered through Pall Microsep Advance centrifugal devices (molecular weight cut-off: 30 kDa; VWR, Budapest, Hungary). Since albumin is a large macromolecule (66.5 kDa), the filter retains HSA and albumin-bound ligands; therefore, the displacement of the site markers by polyphenols results in their elevated concentrations in the filtrate. Using our previously reported methods [50,51], the concentrations of warfarin and naproxen in the filtrate were quantified by HPLC-FLD and HPLC-UV, respectively. HPLC analyses were performed with a Jasco HPLC system (Tokyo, Japan), containing a binary pump (PU-4180), an autosampler (AS 4050), a fluorescence detector (FP-920), and an UV-Vis detector (UV-975). Chromatographic data were evaluated by the ChromNav2 software.

2.5. CYP assays

To investigate the *in vitro* inhibitory effects of RES and its metabolites on CYP enzymes, CypExpress Cytochrome P450 (2C9, 2C19, 2D6, and 3A4) human kits were applied using the FDA-recommended substrates (diclofenac, S-mephenytoin, dextrometorphan, and testosterone) and inhibitors (sulfaphenazole, ticlopidine, quinidine, and ketoconazole), in the absence and presence of polyphenols (0–30 μM). CYP assays were performed as it has been reported, without modifications [42,52].

After incubations, both substrates and metabolites were quantified by HPLC-UV, using the same instrumentation described in Section 2.4. The previously reported HPLC assays were applied with some modifications (because of the co-elution of certain polyphenols with the sunbstrates or metabolites) [42,52].

Method 1: Diclofenac and 4′-hydroxydiclofenac (CYP2C9 assay) were quantified using the following conditions. Guard column: Security Guard (C8, 4.0 × 3.0 mm; Phenomenex, Torrance, CA, US); analytical column: Mediterranea Sea8 (C8, 150 × 4.6 mm, 5 μm; Teknokroma, Barcelona, Spain); eluent: phosphoric acid (6 mM) and ACN (48:52 v/v %); flow rate: 1.0 mL/min; injected volume: 20 μL; detection: 275 nm.

Method 2: S-mephenytoin and 4-hydroxymephenytoin (CYP2C19 assay) were quantified for RES and R3G using the following conditions. Guard column: Phenomenex Security Guard (C8, 4.0 ×3.0 mm); analytical column: Phenomenex Luna (C8, 150 × 4.6 mm, 5 μm); eluent: sodium acetate buffer (6.9 mM, pH 4.0) and ACN (72:28 v/v%); flow rate: 1.0 mL/min; injected volume: 20 μL; detection: 230 nm.

Method 3: Inhibitory effect of R3S on CYP2C19 was tested using the following conditions. Guard column: Phenomenex Security Guard (C8, 4.0 × 3.0 mm); analytical column: Teknokroma Mediterranea Sea8 (C8, 150 × 4.6 mm, 5 μm); eluent: MeOH, water, and acetic acid (53:46:1 v/v%); flow rate: 1.0 mL/min; injected volume: 20 μL; detection: 230 nm.

Method 4: Inhibitory effect of DHR on CYP2C19 was tested using the following conditions. Guard column: Phenomenex Security Guard (C8, 4.0 × 3.0 mm); analytical column: Teknokroma Mediterranea Sea8 (C8, 150 × 4.6 mm, 5 μm); eluent: sodium phosphate buffer (10 mM, pH 4.55), ACN, and MeOH (62:24:14 v/v%); flow rate: 1.0 mL/min from 0.0 to 9.2 min, 1.2 mL/min from 9.2 to 11.1 min, 1.0 mL/min from 11.1 to 12.0 min; injected volume: 20 μL; detection: 230 nm.

Method 5: Dextromethorphan and dextrorphan (CYP2D6 assay) were quantified using the following conditions. Guard column: Phenomenex Security Guard (C8, 4.0 × 3.0 mm); analytical column: Agilent Eclipse XDB C8 (150 × 4.6 mm, 5 μm; Agilent Technologies, Santa Clara, CA, US); eluent: sodium acetate buffer (6.9 mM, pH 4.0) and ACN (72:28 v/v %); flow rate: 1.0 mL/min; injected volume: 20 μL; detection: 280 nm.

Method 6: Testosterone and 6β-hydroxytestosterone (CYP3A4 assay) were quantified for RES, R3G, and DHR as described, with minor modifications [24]. Guard column: Phenomenex Security Guard (C18, 4.0 × 3.0 mm); analytical column: Kinetex EVO-C18 (C18, 150 × 4.6 mm, 5 μm; Phenomenex); eluent: MeOH, water, and acetic acid (53:46:1 v/v%); flow rate: 1.2 mL/min; injected volume: 20 μL; detection: 240 nm.

Method 7: Inhibitory effect of R3S on CYP3A4 was tested using the following conditions. Guard column: Phenomenex Security Guard (C18, 4.0 × 3.0 mm); analytical column: Kinetex EVO-C18 (C18, 150 × 4.6 mm, 5 μm; Phenomenex); eluent: sodium acetate buffer (6.9 mM, pH 4.0) and MeOH (50:50 v/v%); flow rate: 1.2 mL/min; injected volume: 20 μL; detection: 240 nm.

The major validation parameters (including linearity, limit of detection, limit of quantification, and intraday repeatability data) of the HPLC-UV methods used are demonstrated in Table S1.

2.6. OATP assays

A431 cells overexpressing OATP1A2, OATP1B1/3 or OATP2B1, or their mock transfected controls were generated previously [53,54], and were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, Thermofisher 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 °C and 5% CO_2. For cell counting and subculture, the cells were dispersed with a solution of 0.2% trypsin.

Inhibitory effects of RES and its metabolites on OATP1A2, OATP1B1/3, or OATP2B1 were analyzed by measuring the uptake of the fluorescent substrates sulforhodamine 101 (SR101; OATP1A2), 6,8-dihydroxy-1,3-disulfopyrene (OATP1B1 and OATP1B3), or pyranine (OATP2B1) into A431 cells overexpressing the respective OATP as described previously [54,55]. Briefly, A431 cells were seeded on 96-well plates in a density of 80,000 cells per 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 at room temperature with 200 μL phosphate-buffered saline (PBS, pH 7.4). The cells were then preincubated for 5 min at 37 °C with 50 μL Hank's Balanced Salt Solution (HBSS; pH 7.4) or uptake buffer (pH 5.5; OATP2B1 [53]) with or without increasing concentrations (0.7–50 μM) of the compounds tested. The reaction was started with the addition of 50 μL HBSS containing SR101 (0.5 μM, OATP1A2), disulfopyrene (10 μM, OATP1B1

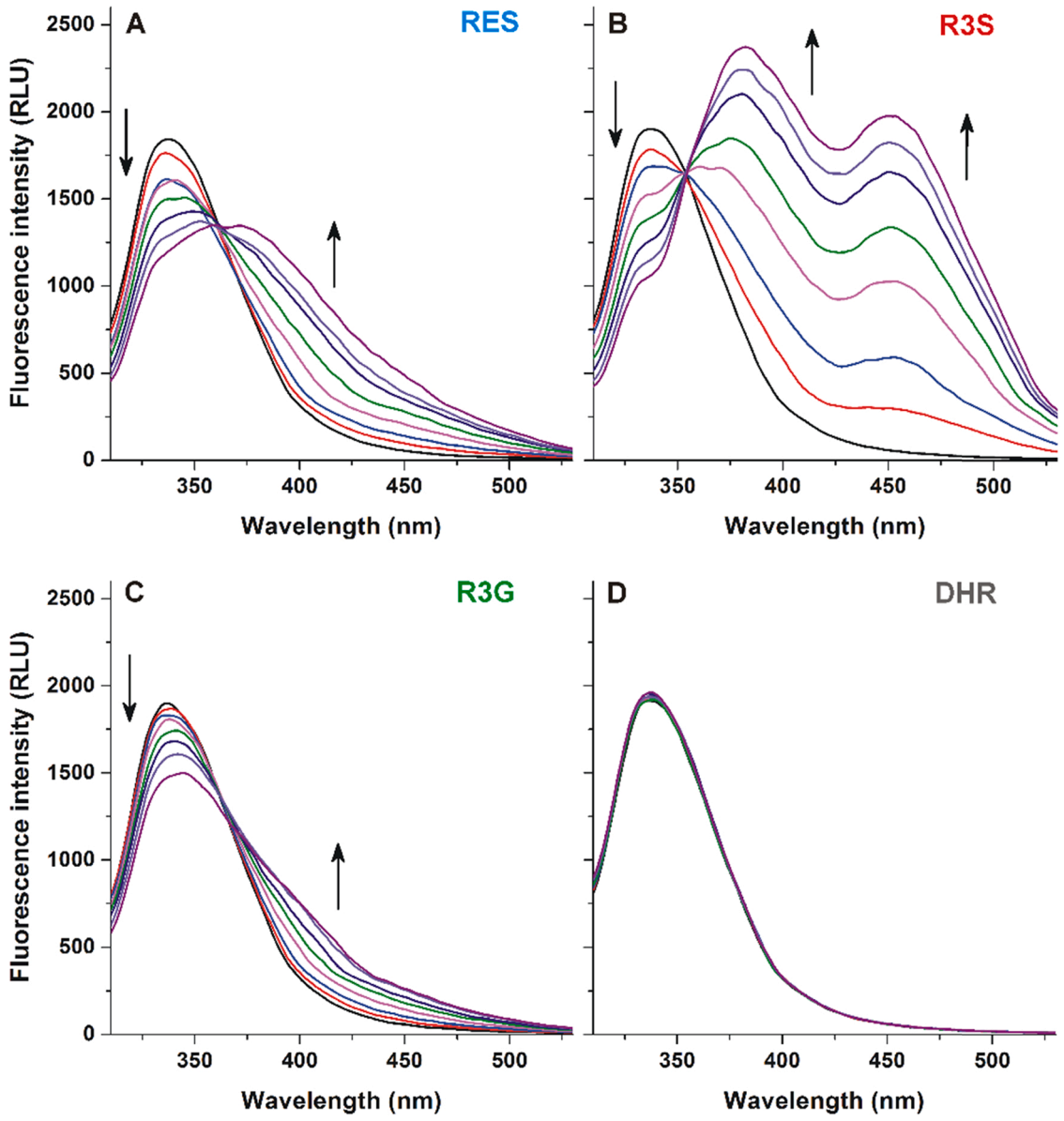

Fig. 2. Representative fluorescence emission spectra of HSA (2 μM) in the presence of increasing concentrations (0, 0.5, 1, 2, 3, 4, 5, and 6 μM) of resveratrol (RES), resveratrol-3-sulfate (R3S), resveratrol-3-glucuronide (R3G), and dihydroresveratrol (DHR) in PBS (pH 7.4; λ_{ex} = 295 nm).

and OATP1B3) or 50 μL uptake buffer containing pyranine (20 μM, OATP2B1). After 15 min (OATP1A2, OATP1B3, and OATP2B1) or 10 min (OATP1B1) incubation at 37 °C, the reaction was stopped by removing the supernatant and washing the cells three-times with 200 μL ice-cold PBS. Fluorescence was determined in 200 μL PBS, employing an Enspire plate reader (Perkin Elmer, Waltham, MA, US) with excitation/emission wavelengths of 460/510 nm (disulfopyrene and pyranine) or 586/605 nm (SR101).

OATP-dependent transport was calculated by extracting the fluorescence measured in mock transfected cells. Transport activity measured in the absence of the compounds tested was set to 100%.

2.7. Data analyses and statistics

Data represent mean and standard error of the mean (± SEM) values, at least from three independent experiments. IC_{50} values were calculated by sigmoidal fitting (Hill1) using the Origin software (version 2018, OriginLab Corporation, Northampton, MA, US). Statistical evaluation ($p < 0.05$ and $p < 0.01$) was established applying one-way ANOVA (and Tukey post-hoc) test with SPSS Statistics software (IBM, Armonk, NY, US).

3. Results

3.1. Testing the interaction of RES and its metabolites with human serum albumin

To test the interactions of RES and its metabolites with HSA, fluorescence quenching studies were performed. Emission spectra of HSA without and with increasing concentrations of RES or its metabolites were collected (λ_{ex} = 295 nm). After the correction of the inner filter effect, complex formations were evaluated based on the concentration-dependent decrease in the emission signal of albumin at 340 nm. DHR did not affect the emission signal of HSA, while RES, R3S, and R3G induced concentration-dependent decrease in the fluorescence at 340 nm (Fig. 2). RES, R3S, and R3G exert intrinsic fluorescence, leading to the appearance of further emission peaks at higher wavelengths which showed some overlap with the emission spectrum of HSA (Fig. 2).

Table 1
Decimal logarithmic values of Stern-Volmer quenching constants (K_{SV}; L/mol) and binding constants (K; L/mol) of polyphenol-HSA complexes based on quenching studies and albumin-induced fluorescence enhancement, respectively (n = 3).

Complex	logK_{SV} ± SEM *quenching*	log$K$ ± SEM *FL enhancement*
RES-HSA	4.80 ± 0.06	5.03 ± 0.05
R3S-HSA	5.04 ± 0.06	5.30 ± 0.05
R3G-HSA	4.25 ± 0.02	4.80 ± 0.03

Stern-Volmer plots of RES, R3S, and R3G (Fig. S1) showed good linearity (R^2 = 0.993–0.997), K_{SV} values suggest that R3S forms the most stable complex wit HSA, followed by RES and R3G (Table 1). Nevertheless, considering the spectral interferences between the emission peaks of HSA and the ligands tested, we felt reasonable to also evaluate the interactions with another model.

In the following experiment, we examined the HSA-induced increase in the emission signals of RES, R3S, and R3G. Increasing amounts of HSA were added to standard polyphenol concentrations in PBS (pH 7.4), then emission spectra were recorded (λ_{ex} = 320 nm). Since HSA showed emission signal at the wavelengths used for the evaluations, background

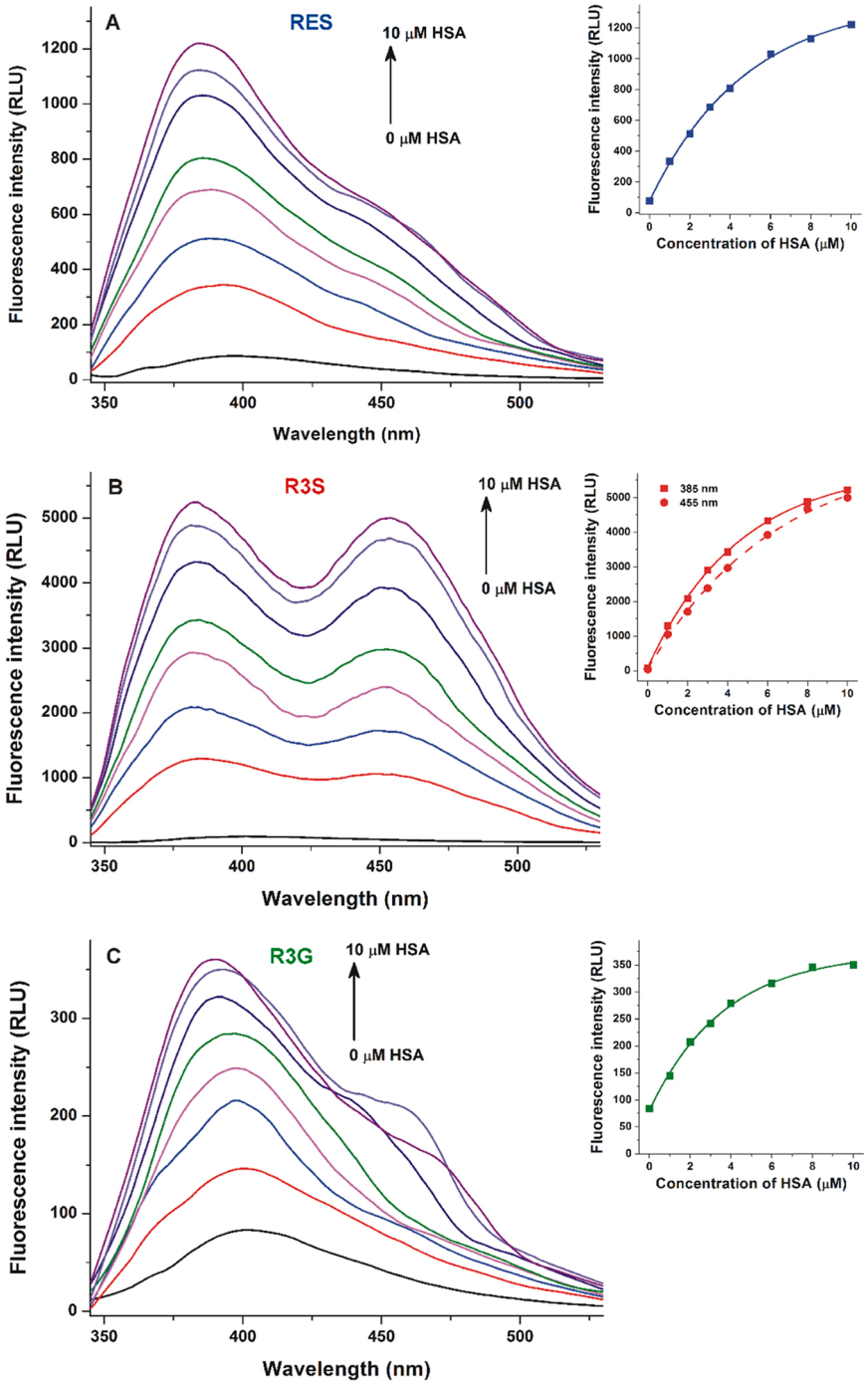

Fig. 3. HSA-induced increase in the intrinsic fluorescence of RES (A), R3S (B), and R3G (C). Representative emission spectra of polyphenols (each 1 μM) in the presence of increasing concentrations of HSA (0, 1, 2, 3, 4, 6, 8, and 10 μM) in PBS (pH 7.4). Emission spectra and intensities were corrected with the background fluorescence of HSA (λ_{ex} = 320 nm for each polyphenol; λ_{em} = 385 nm for RES, 385 nm and 455 nm for R3S, and 395 nm for R3G).

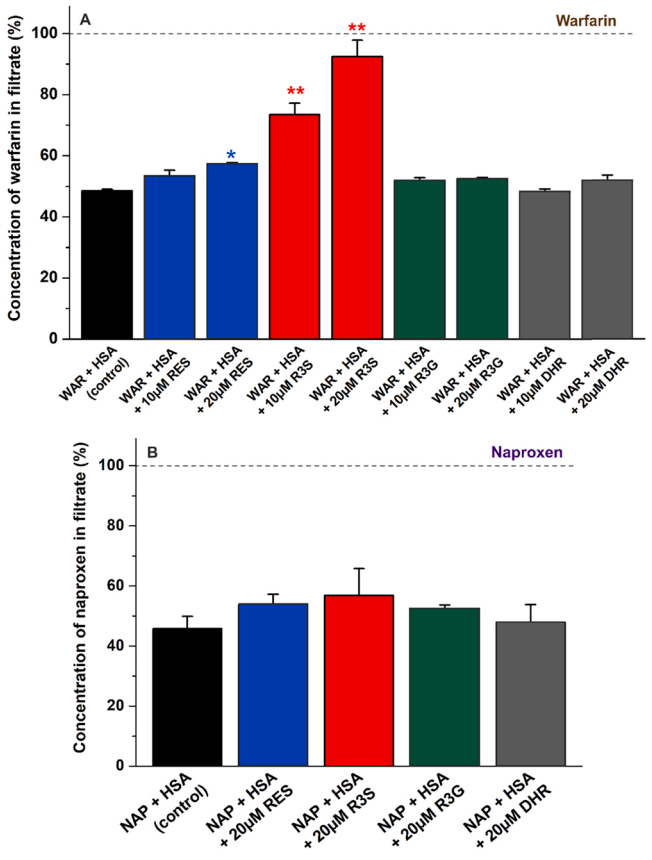

Fig. 4. Effects of polyphenols on the site marker concentrations of filtrates. RES and its metabolites (10 and/or 20 μM) were added to warfarin+HSA (1 and 5 μM, respectively) or naproxen+HSA (1 and 1.5 μM, respectively) combinations in PBS (pH 7.4). After ultrafiltration, the concentrations of warfarin (WAR; site I) and naproxen (NAP; site II) were quantified in the filtrates by HPLC. Dashed line (100%) demonstrates the concentrations of warfarin and naproxen in the filtrate when the samples contained only the site markers (without HSA) (*$p < 0.05$, **$p < 0.01$; $n = 3$). The positive controls for site I (phenylbutazone, 10 μM; concentration of warfarin in filtrate: 82.7%) and site II (ketoprofen, 10 μM; concentration of naproxen in filtrate: 69.5%) caused significant elevation in the filtered fraction of the site markers ($p < 0.01$).

spectra for each albumin concentration were also collected. After background correction, albumin-ligand interactions were evaluated at the emission wavelength maxima of the complexes formed. HSA induced concentration-dependent increase in the emission signals of polyphenols (Fig. 3). RES-HSA (Fig. 3A) and R3G-HSA (Fig. 3C) complexes showed their emission wavelength maxima at 385 nm and 395 nm, respectively. While R3S showed two peaks with an emission maximum at 385 nm and a slightly lower second peak at 455 nm (Fig. 3B). Binding constants were determined using the Benesi-Hildebrand plot (Fig. S2). These data confirm that R3S forms the most stable complex with HSA followed by RES-HSA then R3G-HSA (Table 1).

To confirm the results of fluorescence spectroscopic studies, HPAC experiments were also performed with RES, R3S, and R3G employing a Chiralpak HSA affinity column. The stronger interaction of a ligand molecule with the protein results in its slower elution and consequently its longer retention time (t_R) [49]. R3G was rapidly eluted form the affinity column with $t_R = 1.87$ min. However, considerably longer t_R values of RES (4.51 min) and R3S (4.71 min) were noticed (see the representative HPAC chromatograms in Fig. S3).

In ultrafiltration experiments, the displacing abilities of polyphenols were tested *vs.* the Sudlow's site I and Sudlow's site II ligands warfarin and naproxen, respectively. Since albumin and albumin-bound molecules cannot pass through the filter used, the increased concentrations of site markers in the filtrate indicate their displacement from the protein [50,51]. R3G and DHR did not affect the filtered concentration of warfarin, and RES caused only a slight increase only at 20 μM concentration (Fig. 4A). However, R3S induced a strong, concentration-dependent increase regarding the filtered fraction of the site I marker (Fig. 4A). The polyphenols tested did not affect the filtered fraction of naproxen, even at 20-fold concentration (20 μM) *vs.* the site marker (Fig. 4B).

3.2. Testing the inhibitory effects of RES and its metabolites on CYP enzymes

In the first experiments, high concentrations (20 μM) of RES and its metabolites were tested. The compounds which did not produce at least 50% inhibition under the applied conditions were considered as weak inhibitors.

RES and its metabolites showed only weak inhibitory effects on the CYP2C9-catalyzed diclofenac hydroxylation (Fig. 5A). RES caused approximately 20% decrease in product formation, sulfate and glucuronide conjugates induced even lower inhibition than the parent compound, while DHR did not affect significantly the metabolite formation.

RES caused almost 60% decrease in CYP2C19-catalyzed mephenytoin hydroxylation; while statistically significant but much weaker impacts were produced by R3S, R3G, and DHR (Fig. 5B).

Each polyphenol induced statistically significant but only weak inhibitory impacts on the CYP2D6-catalyzed dextromethorphan demethylation (Fig. 5C). R3G caused the strongest (approximately 30%) inhibition, followed by RES, R3S, and DHR.

R3S and DHR proved to be weak inhibitors of the CYP3A4-catalyzed testosterone hydroxylation (Fig. 5D). However, RES and R3G strongly decreased (approximately 70% and 65%, respectively) the metabolite formation.

The concentration-dependent inhibitory actions of RES on CYP2C19 as well as RES and R3G on CYP3A4 are demonstrated in Fig. 6. Based on these data, the IC_{50} values were determined by sigmoidal fitting. IC_{50} values of these polyphenols and the FDA-suggested positive controls are listed in Table 2. RES is a somewhat weaker inhibitor of CYP2C19 than the positive control ticlopidine; however, their IC_{50} values (10.7 μM and 6.5 μM, respectively) were in the same range. IC_{50} of RES and R3G on CYP3A4 were 9.5 μM and 9.4 μM, respectively; which are considerably higher compared to the positive control ketoconazole (0.12 μM).

3.3. Testing the inhibitory effects of RES and its metabolites on OATP transporters

The inhibitory impacts of RES and its metabolites on the function of multispecific OATPs were tested on A431 cells overexpressing one of the OATPs (1A2, 1B1, 1B3, or 2B1) or mock transfected control using the fluorescent dye substrates SR101 (OATP1A2, [54]), disulfopyrene (OATP1B1 or OATP1B3 [55],) or pyranine (OATP2B1 [53]). DHR showed only weak inhibition of OATP function with only 20% inhibitory impacts of OATP1A2 and OATP1B3 transport at 50 μM, and with IC_{50} values above 25 μM for OATP1B1 and OATP2B1 (Fig. 7 and Table 3). RES inhibited each OATP tested; however, it was only a weak inhibitor of OATP1A2. R3G proved to be a weak inhibitor of OATPs 1A2, 1B1, and 1B3; while it showed similarly strong inhibitory action on OATP2B1 to RES. Finally, R3S induced similar (OATP1B1 and OATP1B3) or stronger (OATP1A2 and OATP2B1) inhibitory effects on OATPs examined than the parent compound RES, with the most pronounced impact on OATP2B1 (Fig. 7 and Table 3).

4. Discussion

RES is a well-known polyphenol commonly contained by dietary supplements. RES metabolites (*e.g.*, R3S and R3G) can reach high concentrations in the circulation and in certain tissues [13–15]; however,

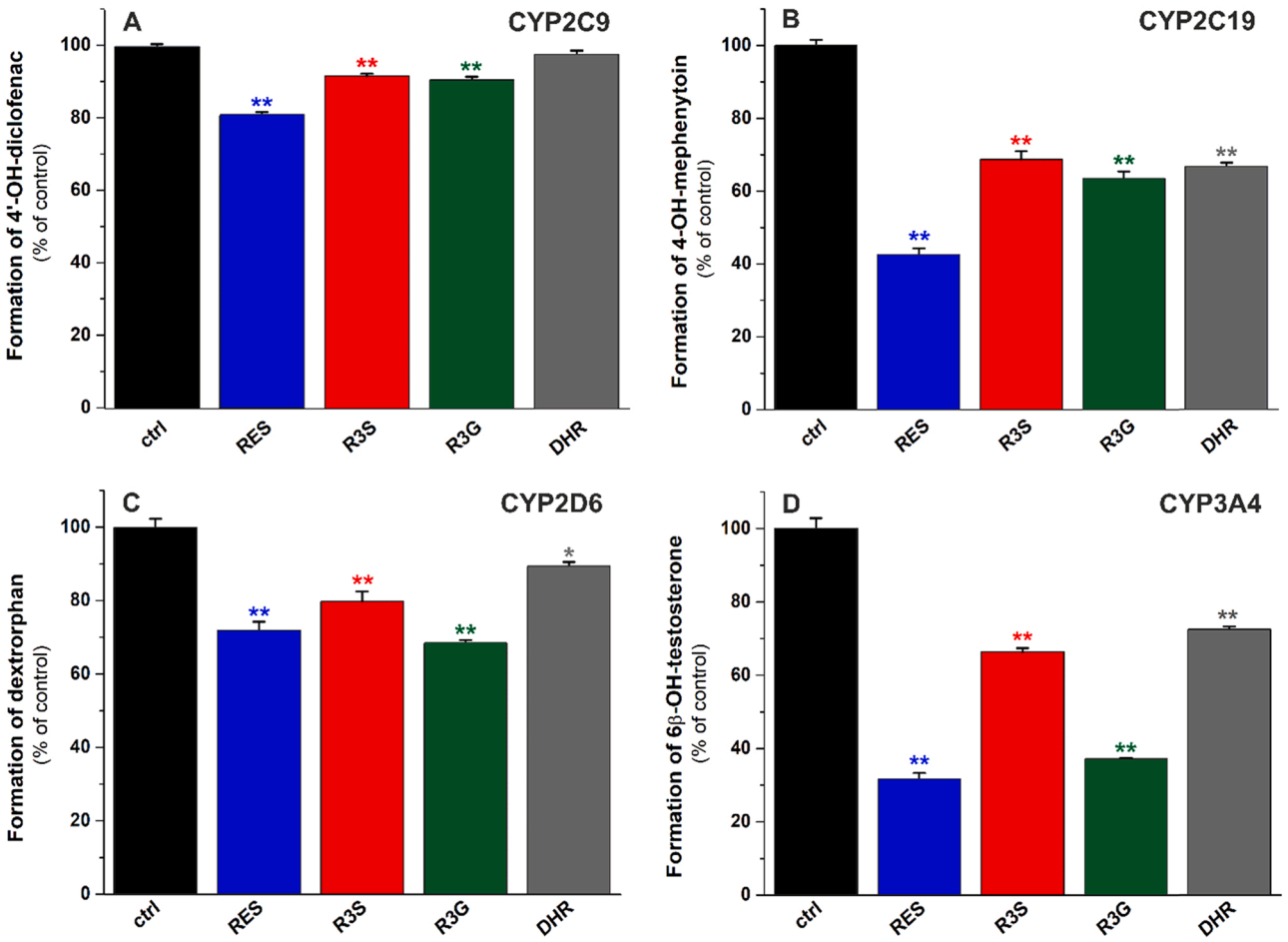

Fig. 5. Inhibitory effects of RES, R3S, R3G, and DHR (each 20 μM) on CYP2C9-catalyzed 4′-hydroxydiclofenac formation (A), CYP2C19-catalyzed 4-hydroxymephenytoin formation (B), CYP2D6-catalyzed dextrorphan formation (C), and CYP3A4-catalyzed 6β-hydroxytestosterone formation (D) (substrate concentrations were 5 μM in each assay; (*$p < 0.05$, **$p < 0.01$; $n = 3$).

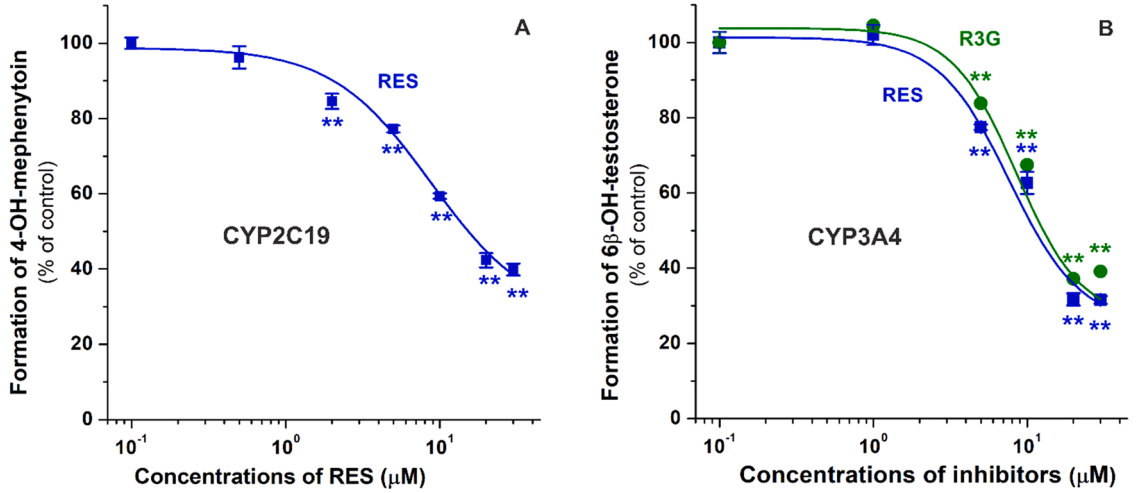

Fig. 6. Concentration-dependent inhibitory effects of RES on CYP2C19 (A) as well as RES and R3G on CYP3A4 (B) (**$p < 0.01$; $n = 3$).

the pharmacokinetic interactions of RES conjugates have been barely characterized. In addition, our recent studies demonstrated that certain microbial metabolites of polyphenols can interact with HSA and/or with certain CYP isoenzymes [29,52]. Thus, the potential impacts of DHR (the colon metabolite of RES [18]) on these proteins can also be interesting. Therefore, in the current study, the interactions of R3S, R3G, and DHR with HSA, CYP (2C9, 2C19, 2D6, and 3A4) enzymes, and OATP (1A2, 1B1, 1B3, and 2B1) transporters were investigated.

HSA is a relatively small protein (66.5 kDa), its sole tryptophan (Trp-214) moiety is mainly responsible for its fluorescence with the minor involvement of tyrosine amino acids. The interaction of ligand molecules with HSA results in the partial quenching of the emission signal of

Table 2
Inhibitory effects of RES and its metabolites on CYP2C19 and CYP3A4 enzymes. Positive controls used: sulfaphenazole (IC_{50} = 2.1 μM, CYP2C9), ticlopidine (IC_{50} = 6.5 μM, CYP2C19), quinidine (IC_{50} = 0.20 μM, CYP2D6), and ketoconazole (IC_{50} = 0.12 μM, CYP3A4).

CYP2C19 assay *(S-mephenytoin hydroxylation)*	**IC_{50} (μM)**
RES	10.7
CYP3A4 assay *(testosterone hydroxylation)*	**IC_{50} (μM)**
RES	9.5
R3G	9.4

Trp-214 suggesting the complex formation [24,50]. In quenching studies, DHR did not affect the emission signal of HSA (Fig. 2D); therefore, it is reasonable to hypothesize that DHR does not form stable complex with the protein. This hypothesis was also supported by ultrafiltration experiments, where DHR did not affect the concentrations of site I or site II markers in the filtrate (Fig. 4). In a concentration-dependent fashion, RES, R3S, and R3G decreased the fluorescence intensity of HSA at 340 nm (Fig. 2), and albumin strongly increased the intrinsic fluorescence of these polyphenols (Fig. 3), demonstrating the formation of polyphenol-albumin complexes. Both logK_{SV} and log$K$ values determined suggest that R3S forms the most stable complex with HSA, followed by RES and R3G (Table 1). These data are in agreement with the results of HPAC studies, where the retention times showed the same order: R3S > RES > R3G. Moreover, in ultrafiltration experiments, R3S strongly displaced the site I marker warfarin from HSA, while RES and R3G have slight and no displacing effects, respectively (Fig. 4). Therefore, it is reasonable to hypothesize that the binding site of R3S is located in site I (subdomain IIA) on HSA. Based on our current knowledge, the interactions of R3S, R3G, and DHR with HSA have not been examined previously. However, earlier studies demonstrated that the parent compound RES forms stable complex with albumin occupying site I as its high-affinity binding site [22–24]. The binding constants determined previously show large variations; however, most studies suggest log$K$ values between 5.0 and 5.8 [22–24].

Table 3
Inhibitory effects of RES and its metabolites on OATP (1A2, 1B1, 1B3, and 2B1) transporters.

	OATP1A2 IC_{50} (μM)	OATP1B1 IC_{50} (μM)	OATP1B3 IC_{50} (μM)	OATP2B1 IC_{50} (μM)
RES	> 25	9.1	11.6	8.3
R3S	16.7	10.1	11.8	3.1
R3G	> 25	> 25	> 25	10.6
DHR	> 25	> 25	> 25	> 25

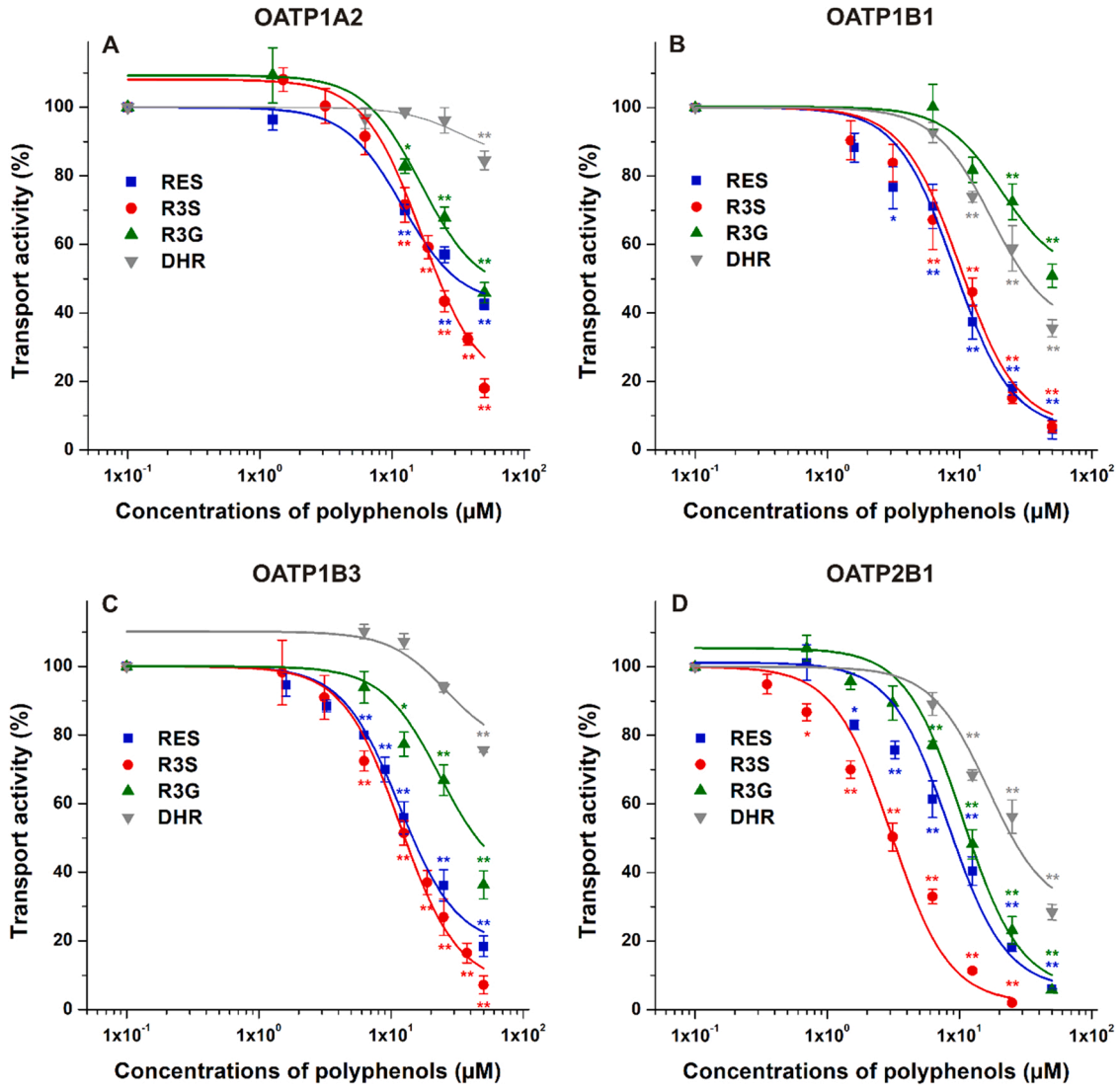

Fig. 7. Concentration-dependent effects of RES and its metabolites on the transport activity of OATP1A2 (A), OATP1B1 (B), OATP1B3 (C), and OATP2B1 (D) (*p < 0.05, **p < 0.01; n = 3).

Nevertheless, the weak displacing effect of RES *vs.* warfarin suggest that the binding constant of RES-HSA is lower compared to the warfarin-HSA complex (log$K$ = 5.4 [56]), which is in agreement with our results (Table 1). Since the consumption of RES-containing dietary supplements can lead to the high concentrations of R3S in the circulation [13–15], the strong displacing effect of R3S *vs.* warfarin suggest that R3S may be able to affect the albumin binding of drugs occupying site I on HSA.

In agreement with our results, the previously reported *in vitro* studies also suggest the moderate-to-weak inhibition of CYP2C9 by RES [24,27,30]. Furthermore, in the current study, R3S and DHR showed slight and no inhibitory effects on this enzyme, respectively (Fig. 5A); which are consistent with the earlier reports of other research groups [27,30]. Interestingly, RES affected the pharmacokinetics of warfarin (in rodents) [57,58], diclofenac [59] and losartan [28] (in healthy human volunteers), suggesting the *in vivo* impacts of RES on CYP2C9 enzyme. Nevertheless, the interaction of RES with serum albumin and/or drug transporters may also be involved.

RES produced considerable inhibition on CYP2C19 enzyme, while R3S, R3G, and DHR caused only weak effects (Fig. 5B). Other *in vitro* studies [27,30] also demonstrated the significant inhibition of CYP2C19 by RES (with similar IC_{50} value reported in this work) and the negligible inhibitory actions of R3S and DHR.

Polyphenols examined proved to be weak inhibitors of CYP2D6-catalyzed dextromethorphan *O*-demethylation (Fig. 5C). In earlier studies, RES showed no or slight inhibitory effects on bufuralol hydroxylation [27,30] and on dextromethorphan *O*-demethylation [29]. In accordance with our results, R3S and DHR did not induce relevant inhibition on bufuralol hydroxylation [27,30]. However, RES caused the strong inhibition of CYP2D6-mediated AMMC (3-[2-(N,N-diethyl-N-methyl-ammonium)ethyl]− 7-methoxy-4-methylcoumarin) demethylation *in vitro* [29], and inhibition of the phenotypic index regarding CYP2D6 (based on the activities measured by the metabolism of dextromethorphan) was described in healthy human volunteers [28].

RES and R3G caused similarly strong inhibition of CYP3A4-catalyzed testosterone hydroxylation, while R3S and DHR produced only weak inhibitory effects (Fig. 5D). Most of the previously reported studies also demonstrated the significant inhibitory action of RES on CYP3A4, showing low micromolar IC_{50} values regarding both testosterone and midazolam hydroxylation [24,27,30]. Furthermore, in earlier reports, R3S did not affect CYP3A4-mediated testosterone hydroxylation [27], while DHR showed considerably weaker inhibition on testosterone hydroxylation than RES but it did not inhibit midazolam hydroxylation [30]. However, RES increased the peak plasma concentrations and AUC values of nicardipine and diltiazem in rodents, due to the inhibition of CYP3A4 and P-glycoprotein [60,61]. In addition, after the repeated *per os* treatment with RES (1 g daily for 4 weeks), the intervention was found to inhibit the phenotypic index of CYP3A4 (based on the activities measured by the metabolism of buspirone) in healthy human volunteers [28]. Our results suggest that both RES and R3G may be involved in regard to the *in vivo* inhibitory action on CYP3A4 enzyme.

OATPs are renowned participants of pharmacokinetics [34,39] and documented victims of drug-drug and food-drug interactions [62]. Previously, RES has been described to inhibit fluorescein-methotrexate transport by OATP1B1 and OATP1B3 [43]. Later, the direct transport of RES and its sulfate and glucuronide metabolites was also investigated [44]. In this latter study, the direct transport of RES by OATPs 1B1, 1B3, and 2B1 was demonstrated; furthermore, R3S proved to be a substrate of OATP1B3. In our study, we confirmed the inhibition of OATPs 1B1 and 1B3 by RES. In addition, we demonstrated that RES can also inhibit the functions of OATP2B1 and OATP1A2, though the latter with much lower potency. We showed here that not only RES, but also R3S and R3G can inhibit hepatic and intestinal OATPs (1B1, 1B3, and 2B1). Comparing the IC_{50} values, we found that OATP2B1 was the most sensitive among the four OATPs investigated, it was inhibited even by DHR. Previous studies suggest the enterohepatic circulation of RES, due to the biliary excretion of both glucuronide and sulfate metabolites [12,13,63]. Therefore, it is reasonable to hypothesize that RES and/or its metabolites may interfere with the OATP-mediated transport of certain drugs: For example, mainly the OATP2B1-dependent drug absorption or the hepatic clearance of OATP1B1/3 substrates can be affected. In addition, R3S may influence the transport through OATP1A2.

Complementary and alternative medicine became popular in the last decades. Dietary supplements containing extremely high doses of RES are widely available on the Internet. The purported positive health effects of RES are commonly applied during the advertisement of these products, while their possible adverse effects as well as the potential pharmacodynamic and pharmacokinetic interactions are typically ignored. Because of the high presystemic elimination of RES, its metabolites can reach much higher concentrations in the circulation and in certain tissues than the parent compound. Many studies described the pharmacokinetic interactions of RES with proteins; however, only few data are available regarding the effects of its metabolites. We examined the interactions of two typical conjugates (R3S and R3G) and a microbial metabolite (DHR) of RES with HSA, CYP enzymes, and OATP transporters. Our results demonstrate that R3S forms highly stable complex with HSA, and can strongly displace the site I marker warfarin from the protein. Furthermore, the strong inhibitory effects of both RES and R3G on CYP3A4 enzyme also underline that not only the parent compound but its metabolites may be involved in the pharmacokinetic interactions caused by RES-containing dietary supplements. In addition, R3S proved to be similarly strong or even stronger inhibitor of OATPs tested than RES, while R3G and RES showed comparable inhibitory actions on OATP2B1. Considering the previous observations that peak plasma concentrations of R3S and R3G can reach or even exceed [13,17] the IC_{50} values determined for certain CYP enzymes and OATP transporters tested in the current study (Tables 2 and 3), these interactions may have clinical relevance. In addition, some *in vivo* studies also demonstrated that RES can alter the pharmacokinetics of certain drugs [16]; therefore, health care professionals should seriously consider the potential hazards of dietary supplement-drug interactions. Our investigations provide novel data and help to understand the mechanism of these interactions, including the involvement of RES metabolites.

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CRediT authorship contribution statement

Miklós Poór: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – original draft; H**ana Kaci:** Formal analysis, Investigation; **Slávka Bodnárová:** Investigation; **Violetta Mohos:** Investigation; **Eszter Fliszár-Nyúl:** Investigation; **Sándor Kunsági-Máté**: Funding acquisition, Resources; **Csilla Özvegy-Laczka:** Conceptualization, Funding acquisition, Methodology, Validation, Writing – original draft; **Beáta Lemli:** Conceptualization, Formal analysis, Investigation.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113136.

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