In vitro investigation of the pharmacokinetic interactions of quercetin and chrysin conjugates

PhD thesis

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1. Introduction, aims

Chrysin (CHR) and quercetin (Q) are common flavonoid aglycones in nature, which occur in several plants, fruits, and vegetables. CHR and Q are also found in many dietary supplements in extremely high doses. Similarly to other flavonoids, the oral bioavailability of CHR and Q is low, due to their poor aqueous solubility and significant presystemic elimination. As a result of the biotransformation of CHR, mainly sulfate (chrysin-7-sulfate: C7S) and glucuronide (chrysin-7-glucuronide: C7G) conjugates are formed. During the metabolism of Q, methyl (isorhamnetin: IR, tamarixetin: TAM), sulfate (quercetin-3'-sulfate: Q3'S), and glucuronide (quercetin-3-glucuronide: Q3G, isorhamnetin-3-glucuronide: I3G) conjugates are produced. With a normal diet, the plasma concentrations of flavonoids and their conjugates are in the nanomolar range, while after their extremely high intake, the total flavonoid (the aglycone and its metabolites) plasma concentrations can reach even more μmol/L.

Human serum albumin (HSA) is the most abundant plasma protein in the circulation. Besides its other functions, HSA is responsible for binding and transporting several drugs in the circulation, which can affect some of the pharmacokinetic properties of these compounds. The cytochrome P450 (CYP) enzymes are a superfamily of enzyme proteins, involved in the biotransformation of numerous drugs. Xanthine oxidase (XO) is a molybdo-flavoprotein, it catalyses the oxidation of hypoxanthine to xanthine, then xanthine to uric acid. Furthermore, the antitumor and immunosuppressant drug, 6-mercaptopurine (6-MP) is also oxidized by XO into the inactive 6-thiouric acid.

Based on previous studies, CHR and Q can interact with certain proteins (e.g. HSA, biotransformation enzymes, drug transporters) in the human organism. However, only limited data are available regarding the pharmacokinetic interactions of the metabolites, which typically reach much higher concentrations in circulation, than the parent compounds. Therefore, in our experiments we aimed to investigate the interactions of CHR and Q conjugates with HSA, CYP enzymes, and XO.

2. Methods

Fluorescence spectroscopic measurements were carried out employing a Hitachi F-4500 fluorimeter. Experiments were performed in PBS (phosphate-buffered saline; pH 7.4), at room temperature. Since the inner filter effects of the test compounds can decrease the fluorescence signal of albumin, the fluorescence spectra were corrected based on the absorbance of flavonoids. Flavonoid-albumin interactions were characterized using fluorescence quenching technique (albumin: 2 μM; flavonoids: 0-5 μM; λ_{ex} = 295 nm, λ_{em} = 340 nm), after which the results were evaluated based on the graphical application of the Stern-Volmer equation and the Hyperquad2006 software. To investigate the displacing ability of flavonoids vs. warfarin (Sudlow's Site I marker) and naproxen (Sudlow's Site II marker), ultrafiltration experiments were performed in PBS, using centrifugal devices with 30 kDa molecular weight cut-off value. The concentrations of warfarin and naproxen in the filtrate were analysed with HPLC-FLD and HPLC-UV, respectively. In addition, changes in the bound fraction of warfarin were also examined employing steady-state fluorescence spectroscopy and fluorescence anisotropy measurements.

The effects of flavonoids and their conjugates on the different CYP enzymes (CYP2C9, 2C19, 2D6, and 3A4) were tested with *in vitro* enzyme assays (using CypExpress™ human enzyme kits). All assays were performed with the FDA (Food and Drug Administration) recommended substrates (CYP2C9: diclofenac, CYP2C19: *S*-mephenytoin, CYP2D6: dextromethorphan, CYP3A4: testosterone) and positive controls (CYP2C9: sulfaphenazole, CYP2C19: ticlopidine, CYP2D6: quinidine, CYP3A4: ketoconazole). Furthermore, solvent controls (DMSO) were also employed in each experiment. After the incubations in a thermomixer, the reactions were stopped with methanol. Then the samples were centrifuged, and the concentrations of substrates and metabolites were analysed in the supernatants with HPLC-UV.

In XO assays, the effects of the flavonoids and their conjugates were tested on the XO-catalysed oxidation of 6-MP, xanthine, and hypoxanthine. All experiments were optimized using 5 μM substrate concentrations. Furthermore, in each assay, allopurinol (APU) was used as positive control. Nevertheless, the effects of its active metabolite (oxipurinol) were also tested. The substrates and the formed metabolites were quantified with HPLC-UV.

Statistical analyses were performed employing one-way ANOVA (with Tukey's post-hoc) test using IBM SPSS Statistics software. To determine IC_{50} values, the metabolite

formation was plotted vs. inhibitor concentrations in a logarithmic scale, using sigmoidal fitting, then data were evaluated employing GraphPad Prism 8 software.

3. Results and discussion

3.1. Interactions of chrysin and its conjugates with serum albumin

In concentration-dependent manner, CHR and its conjugates induced a significant decrease in the fluorescence emission signal of HSA (λ_{ex} = 295 nm, λ_{em} = 340), which indicates the formation of flavonoid-albumin complexes. C7G caused weaker, while C7S produced stronger quenching effect on HSA compared to the parent compound. The fluorescence signal of HSA is mainly exerted by its tryptophan amino acid (TRP214), which is located in Sudlow's Site I. Therefore, considering the strong quenching effects of CHR and its conjugates, it is reasonable to hypothesize that the binding site of flavonoids is located close to TRP214 (in the Site I or close to this region). Furthermore, the Stern-Volmer constants and the binding constants of the flavonoid-albumin complexes showed good correlation. C7S formed the most stable complex with HSA, followed by CHR and C7G.

Previous studies suggest that either Site I or Site II can be the possible binding site of CHR on HSA, therefore, in our ultrafiltration experiments the displacing ability of flavonoids were tested vs. warfarin (Site I marker) and naproxen (Site II marker). Since HSA is a macromolecule (66.5 kDa), albumin and albumin-bound compounds cannot pass through the filter units with a 30 kDa molecular weight cut-off value. Therefore, the increased concentrations of site markers in the filtrate indicates the displacement of warfarin/naproxen from HSA. C7S, at both concentrations applied, induced a remarkable increase in warfarin concentration in the filtrate, causing significantly higher displacement compared to CHR and C7G. Interestingly, the albumin-binding of naproxen was also affected by CHR and its conjugates, although their displacing effects were lower compared to the displacing ability of C7S vs. warfarin. Based on these findings, the high-affinity binding site of C7S is located in Site I. Furthermore, the effect of CHR and its conjugates on the albumin-binding of naproxen can be presumably explained by allosteric interaction.

CHR and its conjugates caused a significant decrease in the emission signal of warfarin-albumin complex (λ_{ex} = 317 nm, λ_{em} = 379 nm). Since the emission intensity of the albumin-bound warfarin is approximately 20-fold higher compared to the free warfarin,

our results suggest the displacement of warfarin from HSA. Based on our results, the displacing ability of flavonoids shows good correlation with their binding affinity towards HSA (C7S > CHR > C7G). In addition, these findings were also supported by the fluorescence anisotropy measurements. Considering the above listed observations, C7S can strongly displace drugs from Site I region of HSA.

3.2. Interactions of chrysin and quercetin conjugates with CYP enzymes

C7G did not inhibit the CYP2C9-catalysed 4'-hydroxydiclofenac formation even at 6-fold higher concentration (30 μM) vs. the substrate. In contrast, CHR (IC_{50} = 3.2 μM) and C7S (IC_{50} = 2.7 μM) proved to be 2.5- and 2-fold weaker inhibitors than the positive control sulfaphenazole (IC_{50} = 1.3 μM), respectively. Therefore, the sulfate conjugate showed even stronger inhibition on CYP2C9 compared to the parent compound. CHR also expressed strong inhibitory effect on CYP2C19, however, C7S and C7G proved to be weak inhibitors. CHR and its conjugates did not influence the CYP2D6-catalysed formation of dextrorphan. Although, CHR was a strong inhibitor of CYP3A4 in previous *in vitro* studies, it showed only weak inhibitory effect in our experiments, and its conjugates exerted only minor inhibition on the enzyme. Based on the scientific literature, after the oral administration of 400 mg single dose of CHR in healthy human volunteers, the peak plasma concentration of C7S (the dominant circulating metabolite of CHR) was 400-800 nmol/L. However, it is very likely that higher doses of CHR can cause micromolar plasma concentrations (similarly to Q). Therefore, it can be hypothesized that high CHR intake may affect the CYP2C9 and/or CYP2C19-mediated biotransformation of some drugs.

The results of the previous studies are often controversial regarding the inhibitory effect of Q on different CYP enzymes. In our experiments, Q and its conjugates showed only weak inhibition on CYP2C19 and CYP3A4 enzymes. Furthermore, Q and its conjugates did not influence CYP2D6. Therefore, it is unlikely that Q can significantly affect the CYP-mediated biotransformation of drugs. Although, it is important to note that similarly to Q, all metabolites tested were significant inhibitors of CYP2C19 and CYP3A4 enzymes. Thus, it is possible that the very high intake of Q may slightly affect these enzymes.

3.3. Interactions of chrysin and quercetin conjugates with xanthine oxidase enzyme

In previous studies, CHR and Q proved to be strong inhibitors of the XO-catalysed oxidation of xanthine. However, some studies report the stronger, while others suggest the

weaker inhibitory effect of these flavonoids compared to the positive control, APU. In our current investigation, CHR, Q, and some Q conjugates (Q3'S, IR, and TAM) exerted similarly strong inhibitory effect on xanthine oxidation to that of APU. However, Q3G, I3G did not inhibit the enzyme, and C7G also showed only weak inhibition.

Interestingly, CHR, Q, and some Q conjugates (Q3'S, IR, TAM) inhibited the oxidation of 6-MP 1.5- to 10-fold stronger than APU. Although, it is important to note that APU inhibited the oxidation of xanthine approximately 5-fold stronger compared to 6-MP oxidation, while the IC_{50} values of CHR, Q, Q3'S, IR, and TAM were similar with both substrates. CHR conjugates showed weaker inhibition on xanthine and 6-MP oxidation compared to the parent compound. However, Q3'S, IR, and TAM proved to be approximately 2- to 7-fold stronger inhibitors of 6-thiouric acid formation, than Q. Among the flavonoids tested, C7S was the only compound, which showed remarkably stronger inhibition on 6-MP oxidation, than uric acid formation. Furthermore, the metabolite formation increased in the presence of higher substrate concentrations, suggesting that flavonoids are reversible inhibitors of XO.

Some authors suggest that flavonoids may be useful in the treatment of hyperuricemia, based on their strong *in vitro* inhibitory effects on XO. However, the results of animal experiments are controversial, and in human studies even high Q doses (2000 mg/day) did not influence the serum uric acid levels. After oral administration of 400 mg single dose of CHR, C7S reached approximately 400-800 nmol/L peak plasma concentrations, while after the repeated daily oral administration of Q (1000 mg/day for 12 weeks), the peak plasma concentrations of total Q (Q and its conjugates) are in the low micromolar range. In contrast, the peak plasma concentrations of APU and oxipurinol are approximately 35-40 μmol/L together (following a single, 200 mg oral dose of APU). However, larger doses can cause even higher concentrations. Thus, the *in vitro* inhibitory effects of flavonoids (and some of their conjugates) on xanthine oxidation are similar to that of APU, while their peak plasma concentrations are much lower compared to APU and oxipurinol. These findings may explain the weak *in vivo* effects of flavonoids regarding the treatment of hyperuricemia.

Since APU inhibits the XO-catalysed elimination of 6-MP, the simultaneous administration of APU with 6-MP can result in toxic consequences. APU and oxipurinol reach much higher concentrations in the circulation (and likely in some tissues as well), than flavonoids, however, some Q metabolites (Q3'S, IR, and TAM) exert considerably stronger inhibition on 6-MP oxidation, than APU. Based on previous human studies, Q3'S

and I3G are the dominant metabolites of Q in the circulation. Furthermore, the high level of I3G in the circulation indicates the significant intracellular formation of IR. Considering these data, the extremely high intake of Q (one or more grams daily intake via dietary supplements) may influence the elimination of 6-MP.

4. Summary

Based on our results, not only the parent compounds, but some of their conjugates can also interact with certain proteins. Moreover, the effects of some conjugates even exceed that of the parent compounds. Although, additional *in vivo* experiments should be performed to confirm the relevance of the listed results, the simultaneous administration of high CHR- and/or Q-containing dietary supplements with drugs (especially drugs with narrow therapeutic window) needs to be carefully considered.

5. New findings

1. Not only CHR, but its conjugates can interact with HSA, we determined the binding constants of the formed complexes. CHR and its conjugates likely occupy Site I on HSA.

2. C7S binds with higher affinity to HSA than CHR, and it also showed stronger displacing ability vs. the Site I marker warfarin.

3. C7G did not or only slightly inhibited the CYP enzymes tested, however, C7S proved to be a potent inhibitor of CYP2C9.

4. Each Q conjugate tested (Q3'S, IR, Q3G, and I3G) produced similar inhibitory effect on CYP2C19 and CYP3A4 enzymes to that of Q.

5. Q3G, I3G, and C7G did not or only weakly inhibited the XO enzyme. Some Q conjugates (Q3'S, IR, and TAM) showed even stronger effects compared to the parent compound.

6. CHR, Q, Q3'S, IR, and TAM produced similarly strong inhibitory effects on xanthine and 6-MP oxidation. APU proved to be a stronger inhibitor of xanthine oxidation, while C7S inhibited 6-MP oxidation with higher potency.

6. List of publications

6.1. Publications related to the present thesis:

Violetta Mohos, Eszter Fliszár-Nyúl, Gabriella Schilli, Csaba Hetényi, Beáta Lemli, Sándor Kunsági-Máté, Balázs Bognár, Miklós Poór, Interaction of chrysin and its main conjugated metabolites chrysin-7-sulfate and chrysin-7-glucuronide with serum albumin. *Int. J. Mol. Sci.* 19 (**2018**) 4073. [**IF: 4.183; Q1**]

Violetta Mohos, Attila Pánovics, Eszter Fliszár-Nyúl, Gabriella Schilli, Csaba Hetényi, Přemysl Mladěnka, Paul W. Needs, Paul A. Kroon, Gábor Pethő, Miklós Poór, Inhibitory effects of quercetin as well as its human and microbial metabolites on xanthine oxidase enzyme. *Int. J. Mol. Sci.* 20 (**2019**) 2681. [**IF: 4.556; Q1**]

Violetta Mohos, Eszter Fliszár-Nyúl, Miklós Poór, Inhibition of xanthine oxidase-catalyzed xanthine and 6-mercaptopurine oxidation by flavonoid aglycones and some of their conjugates. *Int. J. Mol. Sci.* 21 (**2020**) 3256. [**IF: 4.556; Q1**]

Violetta Mohos, Eszter Fliszár-Nyúl, Orsolya Ungvári, Éva Bakos, Katalin Kuffa, Tímea Bencsik, Balázs Zoltán Zsidó, Csaba Hetényi, Ágnes Telbisz, Csilla Özvegy-Laczka, Miklós Poór, 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. *Drug Metab. Dispos.* 48 (**2020**) 1064–1073. [**IF: 3.231; D1/Q1**]

Violetta Mohos, Eszter Fliszár-Nyúl, Orsolya Ungvári, Katalin Kuffa, Paul W. Needs, Paul A. Kroon, Ágnes Telbisz, Csilla Özvegy-Laczka, Miklós Poór, Inhibitory effects of quercetin and its main methyl, sulfate, and glucuronic acid conjugates on cytochrome P450 enzymes, and on OATP, BCRP and MRP2 transporters. *Nutrients* 12 (**2020**) 2306. [**IF: 4.546; D1/Q1**]

Cumulative impact factor of the publications related to the thesis: 21.072

Cumulative impact factor of all papers: 55.433

Number of independent citations: 39

6.2. Oral presentations and posters related to the present thesis:

Violetta Mohos, Attila Pánovics, Miklós Poór, The inhibitory effect of quercetin and chrysin metabolites on xanthine oxidase enzyme by the biotransformation of 6-mercaptopurine and xanthine. ***4th International Cholnoky Symposium*** (Pécs, Hungary, May 10-11, 2018) [**oral presentation**]

Violetta Mohos, Attila Pánovics, Eszter Fliszár-Nyúl, Monika Moravcova, Přemysl Mladěnka, Paul W. Needs, Paul A. Kroon, Miklós Poór, Interaction of human and microbial metabolites of quercetin with serum albumin and biotransformation enzymes. ***5th International Cholnoky Symposium*** (Pécs, Hungary, April 25, 2019) [**oral presentation**]

Miklós Poór, Eszter Fliszár-Nyúl, **Violetta Mohos**, Zelma Faisal, Beáta Lemli, Pharmacological/toxicological importance of albumin-ligand interactions. ***5th International Cholnoky Symposium*** (Pécs, Hungary, May 25, 2019) [**oral presentation**]

Violetta Mohos, Attila Pánovics, Eszter Fliszár-Nyúl, Monika Moravcova, Přemysl Mladěnka, Paul W. Needs, Paul A. Kroon, Miklós Poór, Interactions of human and microbial metabolites of quercetin with serum albumin and biotransformation enzymes. ***4th Symposium on Weak Molecular Interactions*** (Matsue, Japan, May 17-19, 2019) [**oral presentation**]

Miklós Poór, Eszter Fliszár-Nyúl, **Violetta Mohos**, Zelma Faisal, Beáta Lemli, Csaba Hetényi, Sándor Kunsági-Máté, Pharmacological/toxicological importance and investigation of albumin-ligand interactions. ***4th Symposium on Weak Molecular Interactions*** (Matsue, Japan, May 17-19, 2019) [**oral presentation**]

Miklós Poór, **Violetta Mohos**, Eszter Fliszár-Nyúl, Interactions of conjugated and colon metabolites of flavonoids with serum albumin and biotransformation enzymes. ***13th World Congress on Polyphenols Applications*** (Valletta, Malta, September 30- October 01, 2019) [**oral presentation**]

Poór Miklós, **Mohos Violetta**, Fliszár-Nyúl Eszter, Interactions of flavonoid metabolites with serum albumin and biotransformation enzymes. ***XVI. Congressus Pharmaceuticus Hungaricus*** (September 10-12, 2020) [**oral presentation**]

Violetta Mohos, Beáta Lemli, Sándor Kunsági-Máté, Gabriella Boda, Balázs Bognár, Miklós Poór, Interaction of chrysin and its metabolites with human serum albumin. ***12th World Congress on Polyphenols Applications*** (Bonn, Germany, September 25-28, 2018) [**poster**]

Violetta Mohos, Eszter Fliszár-Nyúl, Tímea Bencsik, Balázs Bognár, Miklós Poór, Interactions of chrysin conjugates with cytrochrome P450 enzymes. ***13th World Congress on Polyphenols Applications*** (Valletta, Malta, September 30- October 01, 2019) [**poster**]

Mohos Violetta, Pánovics Attila, Fliszár-Nyúl Eszter, Poór Miklós, Quercetin és chrysin konjugált metabolitjaik kölcsönhatásai xantin-oxidáz enzimmel. ***TOX'2019 Tudományos Konferencia*** (Szeged, Hungary, October 9-11, 2019) [**poster**]

6.3. Other publications:

Miklós Poór, Gabriella Boda, **Violetta Mohos**, Mónika Kuzma, Mónika Bálint, Csaba Hetényi, Tímea Bencsik, Pharmacokinetic interaction of diosmetin and silibinin with other drugs: Inhibition of CYP2C9-mediated biotransformation and displacement from serum albumin. *Biomed. Pharmacother.* 102 (**2018**) 912–921. [**IF: 3.743; Q1**]

Violetta Mohos, Tímea Bencsik, Gabriella Boda, Eszter Fliszár-Nyúl, Beáta Lemli, Sándor Kunsági-Máté, Miklós Poór, Interactions of casticin, ipriflavone, and resveratrol with serum albumin and their inhibitory effects on CYP2C9 and CYP3A4 enzymes. *Biomed. Pharmacother.* 107 (**2018**) 777–784. [**IF: 3.743; Q1**]

Nikolett Szentes, Valéria Tékus, **Violetta Mohos**, Éva Borbély, Zsuzsanna Helyes, Exploratory and locomotor activity, learning and memory functions in somatostatin receptor subtype 4 gene-deficient mice in relation to aging and sex. *GEROSCIENCE* 41, (**2019**) 631–641. [**Q1**]

Eszter Fliszár-Nyúl, **Violetta Mohos**, Tímea Bencsik, Beáta Lemli, Sándor Kunsági-Máté, Miklós Poór, Interactions of 7,8-Dihydroxyflavone with serum albumin as well as with CYP2C9, CYP2C19, CYP3A4, and xanthine oxidase biotransformation enzymes. *Biomolecules* 9 (**2019**) 655. [**IF: 4.082; Q1**]

Balázs Zoltán Zsidó, Mária Balog, Nikolett Erős, Miklós Poór, **Violetta Mohos**, Eszter Fliszár-Nyúl, Csaba Hetényi, Masaki Nagane, Kálmán Hideg, Tamás Kálai, Balázs Bognár, Synthesis of spin-labelled bergamottin: a potent CYP3A4 inhibitor with antiproliferative activity. *Int. J. Mol. Sci.* 21 (**2020**) 508. [**IF: 4.556; Q1**]

Violetta Mohos, Eszter Fliszár-Nyúl, Beáta Lemli, Balázs Zoltán Zsidó, Csaba Hetényi, Přemysl Mladěnka, Pavel Horký, Milan Pour, Miklós Poór, Testing the pharmacokinetic interactions of 24 colonic flavonoid metabolites with human serum albumin and cytochrome P450 enzymes. *Biomolecules* 10 (**2020**) 409. [**IF: 4.082; Q1**]

Eszter Fliszár-Nyúl, **Violetta Mohos**, Rita Csepregi, Přemysl Mladěnka, Miklós Poór, Inhibitory effects of polyphenols and their colonic metabolites on CYP2D6 enzyme using two different substrates. *Biomed. Pharmacother.* 131 (**2020**) 110732. [**IF: 4.545; Q1**]

Zelma Faisal, **Violetta Mohos**, Eszter Fliszár-Nyúl, Kateřina Valentová, Kristýna Káňová, Beáta Lemli, Sándor Kunsági-Máté, Miklós Poór, Interaction of silymarin components and their sulfate metabolites with human serum albumin and cytochrome P450 (2C9, 2C19, 2D6, and 3A4) enzymes. *Biomed. Pharmacother.* 138 (**2021**) 111459. [**IF: 4.545; Q1**]

Eszter Fliszár-Nyúl, Zelma Faisal, **Violetta Mohos**, Diána Derdák, Beáta Lemli, Tamás Kálai, Cecília Sár, Balázs Zoltán Zsidó, Csaba Hetényi, Ádám I. Horváth, Zsuzsanna Helyes, Ruth Deme, Dóra Bogdán, Andrea Czompa, Péter Mátyus, Miklós Poór, Interaction of SZV 1287, a novel oxime analgesic drug candidate, and its metabolites with serum albumin. *J. Mol. Liq.* (**2021**) DOI: 10.1016/j.molliq.2021.115945 [**IF: 5.065; Q1**]

6.4. Other presentations:

Mohos Violetta, Bencsik Tímea, Boda Gabriella, Poór Miklós, Kaszticin, ipriflavon és rezveratrol kölcsönhatásainak vizsgálata szérum albuminnal, valamint CYP2C9 és CYP3A4 biotranszformációs enzimekkel. ***TOX'2018 Tudományos Konferencia*** (Lillafüred, Hungary, October 17-19, 2018) [**poster**]

Mohos Violetta, Pánovics Attila, Fliszár-Nyúl Eszter, Monika Moravcova, Přemysl Mladěnka, Poór Miklós, A colon mikroflóra által képzett quercetin metabolitok kölcsönhatásainak vizsgálata szérum albuminnal, valamint xantin-oxidáz és CYP2C9 biotranszformációs enzimekkel. ***Farmakokinetika és Gyógyszermetabolizmus Szimpózium*** (Galyatető, Hungary, April 10-12, 2019) [**poster**]

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