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# Inhibitory Effect of Acacetin, Apigenin, Chrysin and Pinocembrin on Human Cytochrome P450 3A4

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**Abstract:** Cytochrome P450 3A4 is the most significant enzyme in metabolism of medications. Flavonoids are common secondary plant metabolites found in fruits and vegetables. Some flavonoids can interact with other drugs by inhibiting cytochrome P450 enzymes. Thus, the objective of this study was to determine inhibition kinetics of cytochrome P450 3A4 by flavonoids: acacetin, apigenin, chrysin and pinocembrin. For this purpose, testosterone was used as marker substrate, and generation of the 6 $\beta$ -hydroxy metabolite was monitored by high performance liquid chromatography coupled with diode array detector.  $IC_{50}$  values, inhibition constants, and rates of inhibition were determined.  $IC_{50}$  values ranged between 0.6 and 11.4  $\mu$ M. The strongest inhibitor was chrysin ( $IC_{50}$  0.6  $\mu$ M, inhibition constant 0.6  $\mu$ M, inhibition rate constant 0.065  $\text{min}^{-1}$ , inhibition efficacy 0.108  $\text{min}^{-1} \mu\text{M}^{-1}$ ). Compared to other flavonoids analyzed, chrysin's inhibitory effect can be attributed to the hydrophobic nonsubstituted B ring, as well as rigidity of the structure. When foods rich in chrysin are consumed, e.g. honey and propolis, chrysin can cause food-drug interactions. Further *in vitro* studies are needed to determine the reactive intermediate responsible for inactivation of cytochrome P450 3A4 enzyme, as well as *in vivo* studies to determine possible clinical significance of this inhibition.

**Keywords:** flavonoids, inhibition, cytochromes P450.

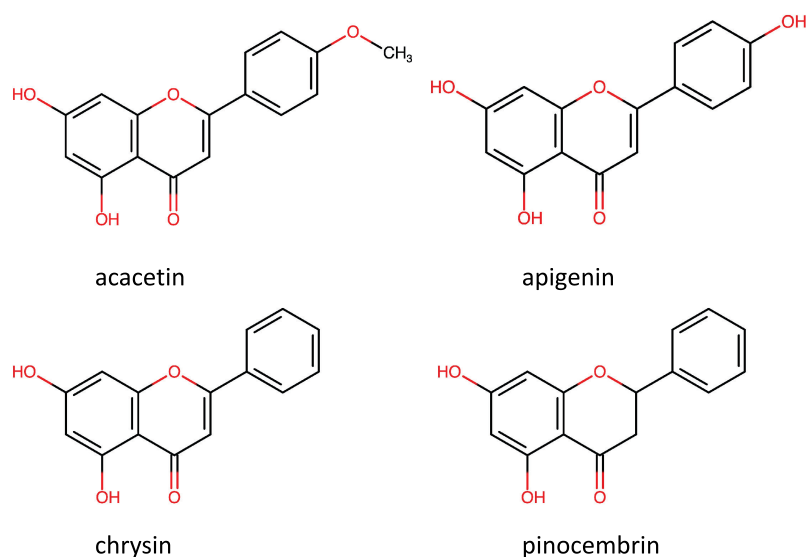
## INTRODUCTION

CYTOCHROMES P450 are the most important metabolic enzymes responsible for over 94 % of metabolic reactions of drugs and other xenobiotics.<sup>[1]</sup> These enzymes do not fit to a lock (enzyme) and key (substrate) enzyme model. Rather, every cytochrome P450 can have numerous substrates and each xenobiotic can be a substrate to multiple cytochrome P450 enzymes.<sup>[2]</sup> Consequently, interactions between different xenobiotics can occur, e.g. drug-drug interactions that can have repercussions on the outcome of the pharmacotherapy as well as possible side-effects.

Most common reason for metabolic drug-drug interactions are inhibitions of cytochromes P450.<sup>[3]</sup> These inhibitions can be reversible, most commonly competitive,

and are regarded as less severe, as an adjustment of the medication dose will usually resolve possible unwanted effects of the interaction.<sup>[3]</sup> However, in the case of irreversible inhibition, a simple adjustment of the dose is not possible, and a drug, the perpetrator of the inhibition, needs to be discontinued and preferably replaced. If an irreversible inhibition is observed, enzyme activity diminishes, and new copies of the enzyme have to be expressed for the enzyme activity to be recovered completely. This process can take from a few days to a few weeks, depending of the metabolic enzyme.<sup>[3]</sup>

While metabolic drug-drug interactions are of significant interest, food-drug interactions have just come into focus.<sup>[4,5]</sup> Flavonoids, as the most common secondary metabolites found in higher plants, are constituents of daily foods.<sup>[6]</sup> These compounds have been extensively studied



**Figure 1.** Structural characteristics of studied flavonoids: flavones acacetin, apigenin and chrysin, and flavanone pinocembrin.

for over a century when they were discovered as vitamin P.<sup>[7]</sup> It has been shown that they have antioxidant, anti-inflammatory, hepatoprotective, antimicrobial, cardioprotective, and other pharmacological properties.<sup>[6,8]</sup> The major issue of their application *in vivo* is their bioavailability. However, it has been shown that some flavonoids, e.g. chrysin, can achieve submicromolar concentrations in plasma and some flavonoids, as soya isoflavones, hesperetin and diosmetin, have been in commercial use as dietary supplements.<sup>[9]</sup>

Cytochrome P450 3A4 is the most significant cytochrome P450 enzyme as it metabolizes about one third of the drugs.<sup>[1,10]</sup> In the previously published screening study, it has been shown that acacetin, apigenin, chrysin and pinocembrin are the most prominent inhibitors of human cytochrome P450 3A4 at 1  $\mu\text{M}$  concentration.<sup>[11]</sup> Flavanone pinocembrin (Figure 1.) reduces the enzyme activity by 50 %, while flavones acacetin, apigenin and chrysin reduce the enzyme activity to 5 %, 24 % and 17 %, respectively.<sup>[11]</sup>

The inhibition of P450 3A4 by acacetin, apigenin, chrysin and pinocembrin is not well characterized, and, if available, is reported as  $IC_{50}$  value. The  $IC_{50}$  values are dependent of the type of inhibition (direct, time and metabolism dependent), as well as experimental setup. Thus, the objective of this study was to determine metabolism dependent inhibition kinetics, inhibition constants and rates of inhibition of P450 3A4 by the aforementioned flavonoids. As these flavonoids have different structural features (Figure 1.): presence or absence of double bond at the position C2-C3,

hydroxylation of B ring and methylation of hydroxyl groups, conclusions about structure-activity relationship can be made.

## EXPERIMENTAL SECTION

### Materials

Flavonoids used in this study (acacetin, apigenin, chrysin, and pinocembrin) were acquired commercially from Sigma-Aldrich (St. Louis, MO, USA).

Recombinant cytochromes P450 3A4 coexpressed with NADPH cytochrome P450 reductase and cytochrome  $b_5$  in baculosomes were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and NADP disodium salt were purchased from Sigma Aldrich. Potassium phosphate (p.a.) and dichloromethane (p.a.) were purchased from Kemika d.d. (Zagreb, Croatia). Methanol for chromatography was purchased from Merck KGaA (Darmstadt, Germany). Ultra-pure water was produced using Arium comfort combined water production system from Sartorius AG (Goettingen, Germany).

### Incubations for Determination of Inhibition Kinetics

To achieve the objective of this study, testosterone was used as the marker substrate. Generation of the 6 $\beta$ -hydroxy metabolite, that reflected residual enzyme activity, was monitored by high performance liquid chromatography coupled with diode array detector (HPLC-DAD).<sup>[12]</sup> For different concentrations of flavonoid, residual

enzyme activity was determined after different periods of incubation. This data was used to construct Michaelis-Menten curve, and inhibition constants and rate of inhibition were determined. The results were assessed in the light of current guidelines on inhibition studies.<sup>[13]</sup>

Evaluation of enzyme kinetics was conducted using baculosomes of recombinant cytochrome P450 3A4 coexpressed with NADPH cytochrome P450 reductase and cytochrome b<sub>5</sub>. A range of flavonoid aglycons concentrations from 0.01 to 20  $\mu\text{M}$  was prepared; appropriate aliquots of 20 mM flavonoid solutions were transferred to glass tubes and evaporated until dry on a water bath equipped with mechanical shaking. After solvent evaporation, a 100  $\mu\text{L}$  incubation mixture was prepared in each of the tubes by adding cytochrome P450 baculosomes (5 pmol), 50 mM potassium phosphate buffer pH 7.4, and ultra-pure water. Generating system containing glucose-6-phosphate, NADP<sup>+</sup> and glucose-6-phosphate dehydrogenase in a ratio 100:50:2 (V/V/V) was used as a source of the coenzyme (15 % volume in final incubation, V/V), and its addition marked the beginning of the reaction. Pre-incubations were conducted in duplicate for zero, 5, 10, 15, 20 and 25 minutes.<sup>[14]</sup>

After the appropriate time period, the residual enzyme activity was tested by adding 1  $\mu\text{L}$  of testosterone solution (final concentration 200  $\mu\text{M}$ ). For determination of  $IC_{50}$  values, preincubation was set to 15 minutes. Testosterone served as the marker substrate of cytochrome P450 activity. The reaction was quenched by adding 1 mL of cold dichloromethane. Reaction tubes were centrifuged at 1900 *g* (3000 rpm) for 10 minutes. The dichloromethane layer was transferred into a HPLC vial, and the organic solvent was evaporated under a stream of nitrogen. Dry residues were dissolved in methanol (30  $\mu\text{L}$ ) and analyzed by HPLC-DAD.<sup>[12]</sup>

### HPLC-DAD Analysis

High performance liquid chromatography coupled with diode array detection (Agilent 1100 instrument, Santa Clara, CA, USA) was used for the analysis of testosterone and the 6 $\beta$ -hydroxy metabolite. A C<sub>18</sub> analytical column (Agilent Zorbax SB C18 column 4.6  $\times$  250 mm, 3  $\mu\text{m}$ ) was used for isocratic analysis with a mixture of 64 % CH<sub>3</sub>OH/36 % H<sub>2</sub>O (V/V) at a flow rate of 1.0 mL/min. Analytes were detected at 240 nm, and the amount of generated 6 $\beta$ -hydroxy testosterone was determined as the area under the curve based on the calibration curve of the standard.<sup>[12]</sup>

### Determination of Enzyme Inhibition Parameters

All incubations were conducted in duplicate. The results were expressed as the amount of generated metabolite based on HPLC-DAD analysis (*vide supra*). Based on these

results, inhibition rates were determined and used for calculation of the major parameters of enzyme inhibition kinetics (inhibition constant and inhibition rate constant) based on the Michaelis-Menten equation. Non-linear three parameters sigmoidal-logistic equation was used for  $IC_{50}$  calculations. Program R (The R Project for Statistical Computing, Vienna, Austria) was used for calculations.

## RESULTS AND DISCUSSION

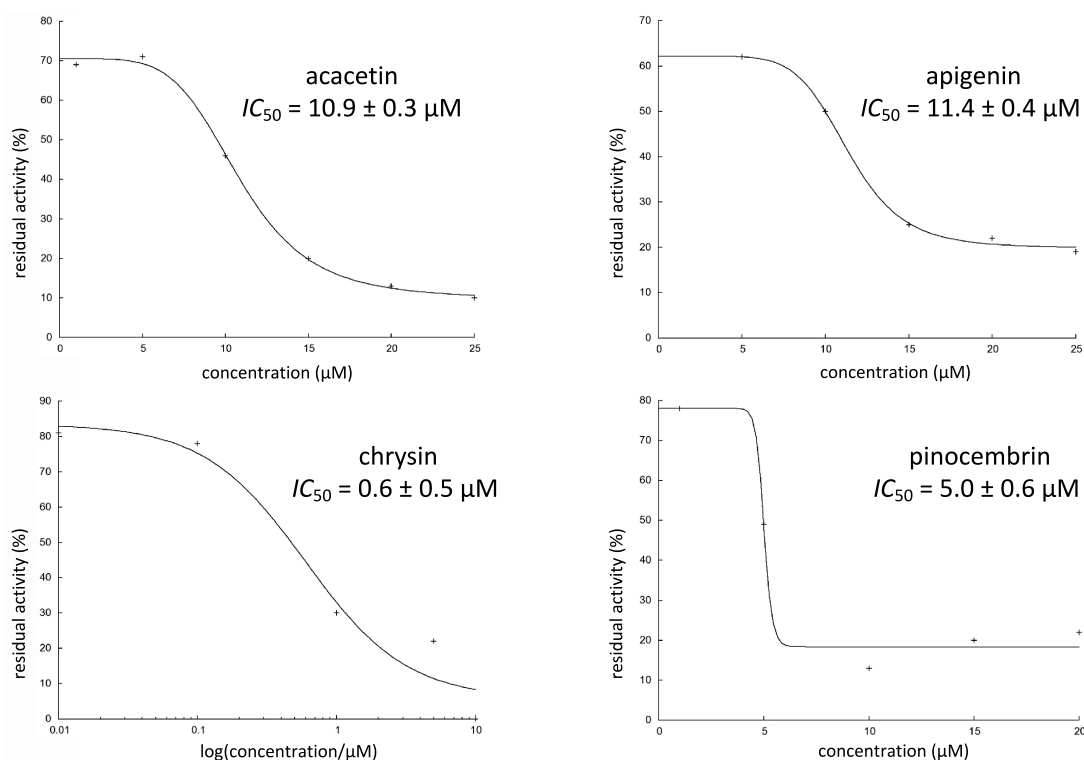
In the previously published screening study, it was shown that acacetin, apigenin, chrysin, and pinocembrin inhibit human cytochrome P450 3A4 at 1  $\mu\text{M}$  concentration.<sup>[11]</sup> Herein, we have characterized the inhibition kinetics and determined  $IC_{50}$  values inhibition constants ( $K_i$ ), and rates of inhibition ( $k_{\text{inact}}$ ) for each of the aforementioned flavonoids. The strongest inhibitor was chrysin ( $IC_{50}$  0.6  $\mu\text{M}$ , inhibition constant 0.6  $\mu\text{M}$ , inhibition rate constant 0.065  $\text{min}^{-1}$ , inhibition efficacy 0.108  $\text{min}^{-1} \mu\text{M}^{-1}$ ).

Since the inhibition was characterized as metabolism-dependent, the first objective herein was to determine the inhibitory concentrations  $IC_{50}$  (Figure 2.). For this purpose, flavonoids were first preincubated with the cytochrome P450 3A4 enzyme for 15 minutes, after which the residual activity was determined using testosterone as the marker substrate.

Chrysin had the lowest  $IC_{50}$  value. This is in agreement with the fact that a 1  $\mu\text{M}$  concentration of chrysin reduced the enzyme activity by 95 %, indicating that the  $IC_{50}$  value is in a submicromolar range.<sup>[11]</sup> Herein, we obtained a value of  $0.6 \pm 0.5 \mu\text{M}$ . Acacetin and apigenin had around twentyfold higher  $IC_{50}$  values,  $10.9 \pm 0.3 \mu\text{M}$  and  $11.4 \pm 0.4 \mu\text{M}$ , respectively. In the previously conducted molecular docking study, it was shown that chrysin has a higher binding affinity to cytochrome P450 3A4 as a neutral molecule, exposing the B ring to the iron in the active center of the enzyme.<sup>[11,15]</sup> This confirms that cytochromes P450 tend to metabolize more lipophilic species.<sup>[9]</sup> By exposing the B ring to the cytochrome P450 active site, a reactive intermediate responsible for inactivation can be generated.

Acacetin and apigenin differ from chrysin as they have a methoxy and a hydroxy group, respectively, at the 4' position of the B ring. Oxygen at the 4' position probably interacts with the ferric ion in the active site of the enzyme and thus tends to inhibit enzyme activity reversibly as a ligand, which has also been reported.<sup>[15]</sup> Consequently, the observed  $IC_{50}$  values for acacetin and apigenin were higher when compared to chrysin.

Interestingly, pinocembrin had around tenfold higher  $IC_{50}$  value of  $5.0 \pm 0.6 \mu\text{M}$  when compared to chrysin ( $0.6 \pm 0.5 \mu\text{M}$ ). Pinocembrin belongs to the flavanone class while chrysin is a flavone, and the only difference in their structures is a single bond at the C2-C3 position in



**Figure 2.** Values of inhibitory concentration that reduces enzyme activity to 50 % ( $IC_{50}$ ) for each of the analysed flavonoids.

pinocembrin vs. a double C2=C3 bond in chrysin. A double bond in chrysin makes the structure rigid, and no energy loss is observed while binding to the active site.

Acacetin is one of the major polyphenols present in honey, which is believed to be associated with the prevention of heart disease.<sup>[16]</sup> Different studies indicate beneficial effects this flavonoid shows. In addition to the prevention of heart disease, its antioxidant, anti-inflammatory and antiparasitic properties have also been shown.<sup>[17,18]</sup> Additionally, studies indicate its antiproliferative properties on different types of tumor cells present in the liver, prostate, and lungs.<sup>[19]</sup> On a rat cytochrome P450 3A subfamily it was determined that acacetin inhibits the enzyme with a  $IC_{50}$  value of 8.2  $\mu\text{M}$ , using midazolam as the marker substrate.<sup>[20]</sup> This is similar to the  $IC_{50}$  value of  $10.9 \pm 0.3 \mu\text{M}$  reported herein.

Apigenin is one of the most present flavonoids in food, especially in parsley.<sup>[21]</sup> As reported in the review by Ross and Kasum<sup>[21]</sup>, *in vitro* studies indicate a significant role of apigenin in the prevention of malignancies and cardiovascular diseases as well as the stimulation of the immune system. The stated antitumor properties of apigenin are evident in inhibiting the growth of melanoma cell cultures, therefore its use in various therapeutic combinations against metastatic melanoma has been studied.<sup>[22]</sup> Apigenin has been shown to inhibit P450 3A4 in

an assay using 7-benzyloxy-4-trifluoromethylcoumarin as the marker substrate with an  $IC_{50}$  value of 1.8  $\mu\text{M}$ .<sup>[23]</sup> Another group obtained an  $IC_{50}$  value of  $31 \pm 8 \mu\text{M}$  for apigenin using 7-benzyloxymethoxy-3-cyanocoumarin as the marker substrate of cytochrome P450 3A4.<sup>[24]</sup> Use of different substrates for determining cytochrome P450 3A4 enzyme activity can explain observed differences in  $IC_{50}$  values between studies. As the cytochrome P450 3A4 has a large active site when compared to other human liver cytochromes P450, use of at least two marker substrates is advisable for the assessment of inhibition kinetics.<sup>[3,13]</sup>

Chrysin is naturally present in honey, but also in various plants and propolis.<sup>[25]</sup> Its anti-inflammatory and antioxidant effects are well documented.<sup>[26,27]</sup> Its chemoprotective effects are increasingly being investigated, and chrysin is believed to exert its effect by inducing apoptosis. Chrysin has shown positive *in vitro* effects on cervical cancer, leukemia, prostate and breast cancer, as well as colon cancer.<sup>[28,29]</sup> Chrysin has been shown to inhibit cytochrome P450 3A4 in an enzyme activity assay using 7-benzyloxymethoxy-3-cyanocoumarin as a the marker substrate, and the obtained  $IC_{50}$  value was  $95 \pm 31 \mu\text{M}$ .<sup>[24]</sup> In contrast, when testosterone was used as the marker substrate,  $IC_{50}$  value was determined to be 0.9  $\mu\text{M}$ , similar to this study ( $0.6 \pm 0.5 \mu\text{M}$ ).<sup>[30]</sup>

Pinocembrin modulates inflammatory responses, and there is a potential for its use in the treatment of ischemic stroke and similar clinical conditions.<sup>[31]</sup> In addition, its antifungal activity has been thoroughly described, as well as the induction of apoptosis in colon cancer cells.<sup>[32]</sup> While its *in vitro* pharmacological effects have been extensively studied, this is not the case with inhibition of cytochrome P450 3A4 enzyme. Herein, we have reported an  $IC_{50}$  value of  $5.0 \pm 0.6 \mu\text{M}$ .

Aforementioned results of inhibition assays have been expressed as  $IC_{50}$  values. As  $IC_{50}$  values are depend on the experimental setup further characterization of inhibition is needed. Thus, we have analyzed inhibition kinetics for each flavonoid, results of which are presented in Figure 3.

While for the most flavonoids the rate of inactivation was assessed at 0, 1, 5, 10, 15 and 20  $\mu\text{M}$  final concentration in flavonoid incubation, due to its low  $IC_{50}$  value, concentrations for chrysin were adjusted to 0.01, 0.1, 1, 5 and 10  $\mu\text{M}$ .

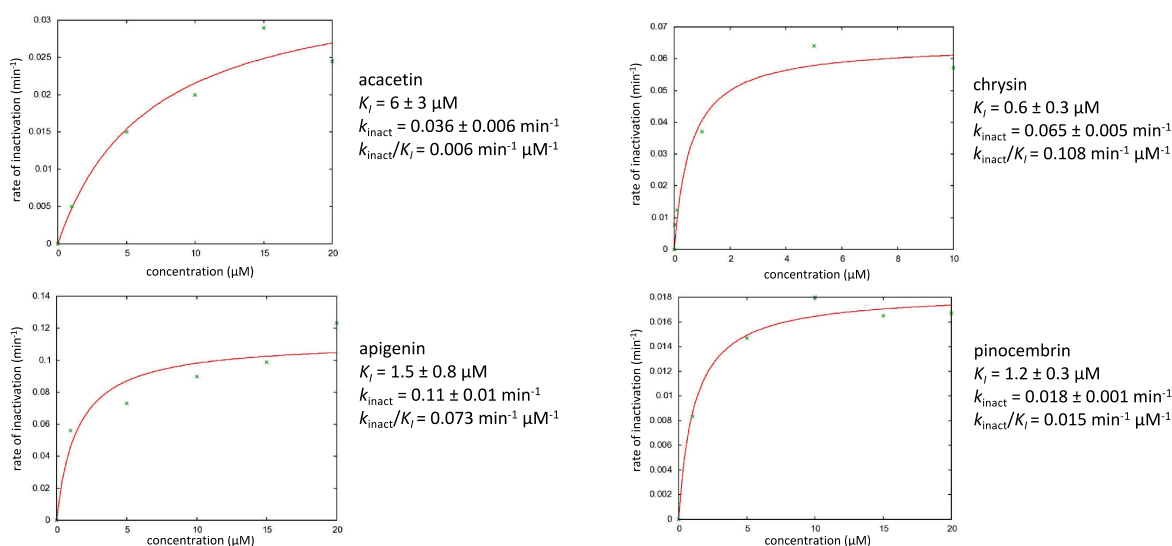
The lowest inhibition constant was found for chrysin, with a value of  $0.6 \pm 0.3 \mu\text{M}$ . A flavanone analogue of chrysin – pinocembrin had a higher value of  $1.2 \pm 0.3 \mu\text{M}$ , with a much lower value of inhibition rate of  $0.018 \pm 0.001 \text{ min}^{-1}$  compared to chrysin ( $0.065 \pm 0.005 \text{ min}^{-1}$ ). Hydroxylated and methoxylated chrysin derivatives, i.e. apigenin and acacetin had higher values of inhibition constant:  $1.5 \pm 0.8 \mu\text{M}$  and  $6 \pm 3 \mu\text{M}$ , respectively, while their inhibition rate constants were  $0.11 \pm 0.01 \text{ min}^{-1}$  and  $0.036 \pm 0.006 \text{ min}^{-1}$ .

Consequently, inactivation efficacy was the highest for chrysin,  $0.108 \text{ min}^{-1} \mu\text{M}^{-1}$ . This was followed by apigenin

( $0.073 \text{ min}^{-1} \mu\text{M}^{-1}$ ), pinocembrin ( $0.015 \text{ min}^{-1} \mu\text{M}^{-1}$ ) and acacetin ( $0.006 \text{ min}^{-1} \mu\text{M}^{-1}$ ). In comparison, one of the well-known time dependent cytochrome P450 3A4 inhibitors, mibefradil, shows an inactivation efficacy of  $0.174 \text{ min}^{-1} \mu\text{M}^{-1}$ .<sup>[33]</sup> This antihypertensive drug has been retracted from market due to clinically significant interactions it caused with over 30 drugs in the market.

Extrapolation of the inactivation kinetic data obtained in the herein study is not straightforward. The setup of the experiment enables us to characterize observed metabolism/time dependent inhibition. Other parameters can also influence the results, e.g. some drugs can act as substrates and inhibitors at the same time.<sup>[3]</sup> However, based on previous reports by Benković *et al.*,<sup>[34,35]</sup> metabolism of acacetin, apigenin, chrysin and pinocembrin mediated by cytochrome P450 3A4 can be disregarded. Acacetin is susceptible to *O*-demethylation and aromatic hydroxylation, generating two products, apigenin and luteolin, which is attributed to cytochromes P450 1A2 and 2D6; apigenin undergoes aromatic hydroxylation to luteolin and this is mediated by cytochrome P450 1A2; chrysin is aromatically monohydroxylated to baicalein or norvogonin or dihydroxylated to luteolin, and this is mediated to some extent by cytochrome P450 1A2; while pinocembrin was not found to be susceptible to the metabolism mediated by hepatic cytochromes P450.<sup>[34,35]</sup>

Usually, observed time-dependent inhibition is at the same time metabolism-dependent, meaning that cytochrome P450 generates a reactive intermediate that inactivates the enzyme (suicide substrates).<sup>[3]</sup> It can be speculated that this could be due to generation of a reactive epoxide on an aromatic ring and further research



**Figure 3.** Inhibition kinetic parameters for each of the analysed flavonoids:  $K_i$  – inhibition constant,  $k_{\text{inact}}$  – inhibition rate constant, and  $k_{\text{inact}}/K_i$  – inactivation efficacy.



should focus on determining the structure of the reactive intermediate. However, this is not an easy task, as the inhibition rate constant of chrysin is  $0.065 \pm 0.005 \text{ min}^{-1}$ . Incubations are usually limited to 30 minutes, as longer incubations can result in hydrogen peroxide formation due to futile catalytic cycles. Hydrogen peroxide is known to destroy the heme moiety in the active site of the enzyme. Consequently, in half an hour, under maximal inhibition rate, in an incubation with 5 pmol of enzyme only 9.75 pmol of the reactive intermediate would be generated. That means that in an incubation in which 10  $\mu\text{M}$  chrysin is present (concentration that enables maximal inhibition rate), around 100 nM of the reactive intermediate would be generated (1 %). Not surprisingly, even for the aforementioned mibefradil, the reactive intermediate has not been trapped nor was its structure described.<sup>[3]</sup>

To put the obtained results into perspective, an assessment parameter  $R$  can be calculated as per Food and Drug Administration guidelines.<sup>[13]</sup> For time-dependent inhibition  $R = (k_{\text{obs}} + k_{\text{deg}}) / k_{\text{deg}}$  where  $k_{\text{obs}} = (k_{\text{inact}} \times 50 \times I_{\text{max}}) / (K_i + 50 \times I_{\text{max}})$ ;  $k_{\text{obs}}$  is the observed inhibition rate constant of the affected enzyme,  $k_{\text{deg}}$  is the apparent degradation rate constant of the affected enzyme ( $0.0138 \text{ h}^{-1}$  for cytochrome P450 3A4)<sup>[36]</sup>,  $K_i$  is inhibition constant – the inhibitor concentration causing half-maximal inactivation ( $0.6 \mu\text{M}$ ),  $k_{\text{inact}}$  is the inhibition rate constant ( $0.065 \text{ min}^{-1}$ ), and  $I_{\text{max}}$  is the maximal unbound plasma concentration of the interacting drug at steady state ( $12 \text{ nM}$  for chrysin)<sup>[9]</sup>. Thus, assessment parameter  $R$  has a value of 142, well above the threshold of 1.25, and consequently further assessment and pharmacokinetic modeling is needed to evaluate if this inhibition is clinically significant.

Based on the inhibition kinetic data, it can be assumed that interactions with herein studied flavonoids will be clinically significant at the level of metabolic enzyme cytochrome P450 3A4. *In vivo* data on apigenin in animal models confirm this observation.<sup>[37,38]</sup> The inhibition effect of apigenin against cytochrome P450 3A4 mediated metabolism was confirmed in rats when combined with etoposide. Etoposide is metabolized primarily by cytochrome P450 3A4 and, in the presence of apigenin, bioavailability of oral etoposide in rats was increased. This interaction does not necessarily need to be unwanted, as combined use of apigenin might be helpful to improve etoposide bioavailability in chemotherapeutic applications.<sup>[37]</sup> Imatinib, another chemotherapeutic agent, is metabolized by cytochrome P450 3A4 and it has been shown that in the short term apigenin can increase imatinib concentration *in vivo* in rats as an animal model.<sup>[38]</sup>

The major contribution of this study is characterization of inhibition kinetics of cytochrome P450 3A4 by selected flavonoids that show metabolism dependent inhibition i.e. inactivation of the enzyme. Further *in vitro*

studies can be conducted on different enzyme sources (e.g. tissue, liver microsomes) or using other marker substrates of cytochrome P450 3A4 (e.g. midazolam, nifedipine). The data from this study can be used to assess flavonoid-drug or food-drug interactions. While the data suggest that clinically significant interaction can exist, further assessment is needed through pharmacokinetic modeling or *in vivo* models to confirm the relevance of the results.<sup>[13]</sup>

## CONCLUSION

Acacetin, apigenin, chrysin and pinocembrin cause time-dependent inhibition of cytochrome P450 3A4.  $IC_{50}$  value is the lowest for chrysin, indicating that hydrophobicity of the nonsubstituted B ring, as well as rigidity of the structure (absence of a single bond between C2 and C3 atoms), plays an important role in the inhibition. Based on the data for inactivation kinetics, it can be concluded that chrysin has the highest potential to cause food-drug interactions when used with foods rich in this flavonoid, e.g. honey and propolis. Further *in vitro* studies are needed to determine the reactive intermediate responsible for inactivation of cytochrome P450 3A4 enzyme, as well as *in vivo* studies to determine possible clinical significance of this inhibition.

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