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ORIGINAL PAPER

In silico and *in vivo* evaluation of flavonoid extracts on CYP2D6-mediated herb-drug interaction

Zhe Su · Bo Zhang · Wenliang Zhu · Zhimin Du

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Abstract Flavonoid extracts are widely used for preventing and treating ischemic heart disease. However, because many flavonoid extracts have been verified to inhibit CYP2D6 the main metabolic enzyme for the majority of antiarrhythmics and beta-blockers, co-administration of flavonoid extracts with these drugs may cause adverse herb-drug interaction in clinic. Here, we evaluated 43 common flavonoids on CYP2D6 inhibition *in sillico* and four commercial flavonoid extracts *in vivo* on the pharmacokinetics and pharmacodynamics of metoprolol in rats. Surprisingly, we found that the core skeletons of flavonoids instead of their substituents determine the extent of inhibiting CYP2D6 by a flavonoid extract. Isoflavones are less likely to inhibit CYP2D6, compared with other categories of flavonoids. Consistently, co-administration of soy extract that mainly contains isoflavones did not significantly increase plasma concentration of metoprolol and alter the systolic blood pressure of rats. Our results have implication in rationally selecting flavonoid extracts for therapeutic application.

Keywords CYP2D6 · CYP450 inhibition · Flavonoid extract · Herb-drug interaction

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Introduction

Joint use of traditional herbal medicines and western drugs in clinic are accepted worldwide [1, 2]. By possessing outstanding antioxidant properties, flavonoids, a subgroup of the more extended family of polyphenols, confirm their appealing potential for therapeutic applications in ischemic heart disease (IHD) [3]. This makes the herbal extracts rich in such components also suitable for IHD protection [4]. Given the complex pathogenesis of IHD, multi-drug combination therapy appears to be inevitable. As the majority of antiarrhythmics and beta-blockers undergo cytochrome P450 2D6 (CYP2D6)-mediated metabolism, co-administration of flavonoid extract or flavonoid-rich herbal medicine may probably produce so-called herb-drug interaction by inhibiting CYP2D6 [5]. CYP2D6 inhibition may substantially increase the plasma concentration of these drugs and cause the associated adverse drug reactions, which may increase the potential risk in patients. For example, co-administration of metoprolol with diphenhydramine, a potent CYP2D6 inhibitor, leads to a significant alteration in pharmacokinetics, further resulting in the changed systolic blood pressure and heart rate response to metoprolol [6].

Although the beneficial effects of flavonoid extracts have been extensively recognized, the assessment of possible risks after treating pharmacologically with them is also necessary [7, 8]. Recently, many *in vitro* experimental evidences have shown different inhibition effects of flavonoid extracts on CYP2D6 [9–13]. Flavonoids are a superfamily of natural compounds, but only limited skeletal structures of flavonoids can be found in a given extract. For example, soy extract mainly contains isoflavones and grape seed extract majorly consists

of flavans and catechins. We speculate that different core skeletons of flavonoids might explain the diverse CYP2D6 inhibition by flavonoid extracts. Working on this hypothesis, we established a prediction model by using Autodock 4.2 to evaluate the CYP2D6 inhibition potential of common flavonoids, which were divided into four categories according to their core skeletons (Supplementary data). Our results show that isoflavones are more likely to be risk-free of CYP2D6-mediated herb-drug interaction. This finding was further verified by the *in vivo* experiment in that effects on the pharmacokinetics and pharmacodynamics (PK/PD) of co-administered metoprolol by different flavonoid extracts were investigated in rats.

Methods

Chemicals and reagents

Metoprolol and propranolol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soy extract (flavonoid content: 90 %, g/g), grape seed extract (flavonoid content: 95 %, g/g), milk thistle extract (flavonoid content: 80 %, g/g) and ginkgo biloba extract (flavonoid content: 26 %, g/g) were all provided by Shanxi Scipher Biotechonology Co., Ltd. (Shanxi, China). All other reagents are analytical grade.

Animals

Male Wistar rats (180-220 g) were purchased from the Animal Experimental Center of the Harbin Medical University. All procedures involving the use of animals complied with the regulations and protocols of the ethic committees of Harbin Medical University and the US National Institutes of Health's Guide for the Care and Use of Laboratory. Forty five rats were randomly divided into nine groups: metoprolol treated (MPL), soy extract treated (SOY), grape seed extract treated (GS), milk thistle extract treated (MT), ginkgo biloba extract treated (GB), metoprolol and soy extract co-treated (MPL-SOY), metoprolol and grape seed extract co-treated (MPL-GS), metoprolol and milk thistle extract co-treated (MPL-MT), metoprolol and ginkgo biloba extract co-treated (MPL-GB). During the following seven days, metoprolol was orally administered at a dose of 25 mg/kg twice daily, while all the flavonoid extracts were orally given at a dose of 30 mg/kg twice daily. The heart rate and blood pressure were noninvasively measured daily by tail-cuff plethysmography and pneumatic pulse transducer (BP-98, Softron, Japan) in each rat one hour after drug administration at 8 am. Blood sample of 0.5 ml was collected from the tail vein using heparin treated tube one hour after drug intake at 4 pm. The plasma sample was then obtained after centrifugation at 3000 rpm for 10 min. All the plasma samples were frozen at −20 °C until analysis.

Plasma sample preparation and HPLC conditions

Fifty μl plasma with 20 μl internal standard propranolol was deproteinized by adding 130 μl of acetonitrile. After the mixture was vortex mixed for 5 min and centrifuged at 3000 rpm for 10 min, 10 μl of the supernatant solution was injected into the Waters 2010 HPLC system 2010 (Waters, MA, USA) with a fluorescence detector. The wavelengths of excitation and emission wavelength were set at 275 and 305 nm. The separation was performed on a Hypersil-C6H5 column (4.6 mm×250 mm, 5 μm, Elite Co.) The isocratic program was adopted with composition of 20 mM potassium dihydrogen phosphate, acetonitrile and triethylamine (45 : 55 : 0.5, v/v/v) at a flow rate of 1 ml/min. The column temperature was set at 30 °C.

CYP2D6 inhibition prediction model

The SMILES strings of 43 common flavonoids were retrieved and downloaded from PubMed Substance database (Supplementary data). They were classified into four categories according to their core skeletal structures (category 1, flavones and flavonols; category 2, flavanones and dihydroflavonols; category 3, isoflavones; category 4, flavans and catechins). To enrich the structural diversity of each category, the core skeleton of each flavonoid was replaced by redrawing the molecular structure with any substituent unchanged. The software ChemDraw was used for structure transformation and created SMILES strings. The 3D PDB files of all compounds were obtained by applying the online tool "Online SMILES Translator and Structure File Generator". By searching the scientific literature with joint terms "CYP2D6", "inhibition" and "Ki", 119 compounds with experimentally validated inhibition constant Ki were found (Supplementary data). However, only 78 compounds with Ki less than 10 μM were identified as CYP2D6 inhibitors because of their more likeliness to exhibit unfavorable drug-drug interactions in clinic [14]. Similar to the above flavonoids, each CYP2D6 inhibitor (Ki<10 μM) or non-inhibitor (Ki>10 μM) was assigned with its 3D PDB file. To access the possible inhibition of flavonoids to the CYP2D6 protein, AutoDock 4.2, a flexible docking tool, was used to investigate the degree of affinity between a small molecule and the macromolecule of CYP2D6 protein (defined as the Ki of the optimal docking conformation) [15]. The 3D PDB file of human CYP2D6 protein crystal structure (Accession number: 2F9Q) was downloaded from the Protein Data Bank database [16]. The crystal water and small molecular ligand SO4 were removed before docking. Notably, we only considered the enzyme catalytic pocket but the whole CYP2D6 enzyme as the

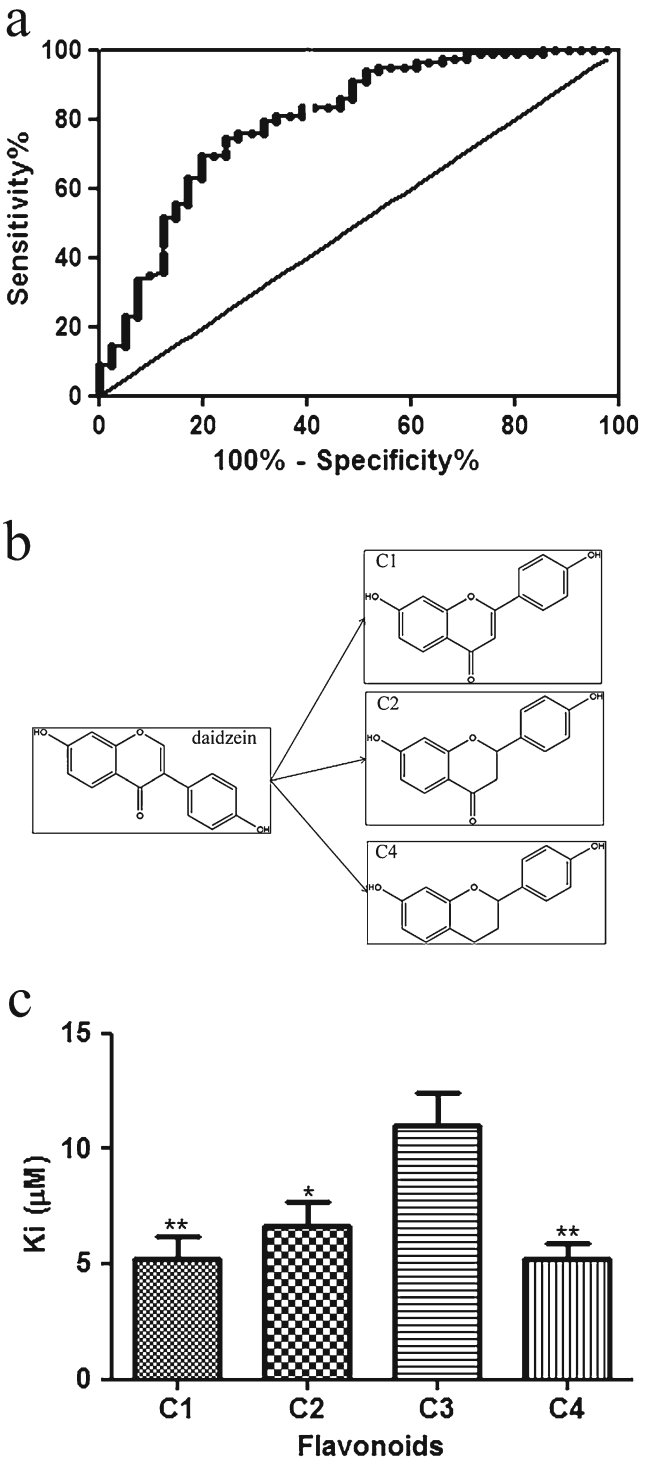

Fig. 1 Results of in sillico evaluation. **a** AOC curve. **b** Daidzein as an example of skeleton transformation. **c** Mean Kis of flavonoids in different categories. C1: Category 1 (flavones and flavonols); C2: Category 2 (flavanones and dihydroflavonols); C3: Category 3 (isoflavones); C4: Category 4 (flavans and catechins). *$p<0.05$ or **$p<0.01$ versus C3 (one-way ANOVA and two-tailed t test)

docking region. The parameter dimension x, y and z were all reset at 40, and the coordinate center x, y and z at -21, 49 and -8, respectively. Other parameters were set as default.

When Autodock 4.2 was used for virtual docking between small molecule and protein, a docked Ki value that represent the affinity energy of binding was calculated by this software. A small docked Ki value means that the small molecule would like steadily bind with protein. In our study, all flavonoids and their corresponding transformed partners underwent docking in Autodock 4.2 for calculating their docked Ki values that were used to estimate the possibility of significant inhibition on CYP2D6. To determine the relationship between the docked Ki values and inhibition constant Ki values, a receiver operating characteristic (ROC)) analysis was performed on the compounds with experimentally validated inhibition constant Ki. After dividing the 119 literature-retrieved compounds into two groups CYP2D6 inhibitors (inhibition constant Ki<10 μM) and CYP2D6 non-inhibitors (inhibition constant Ki>10 μM) according to their known experimentally validated Ki values [14], Autodock 4.2 was used to calculate the docked Ki of each compound with human CYP2D6 protein *in sillico*. Based on their docked Ki values, GraphPad Prism v5.0 was then applied to perform the ROC analysis for establishing a prediction model of potent CYP2D6 inhibitor. The ROC analysis can automatically return an optimal cut-off Ki value that would distinguish CYP2D6 inhibitors from CYP2D6 non-inhibitors best. Most simply, if a flavonoid molecule is assigned with a docked Ki that is less than this value after docking, it is supposed that it can actually inhibit CYP2D6 significantly.

Molecular interaction analysis

Schrodinger instead of Autodock was used for this analysis. The XP (extra precision) high-precision method in the Glide module is used for structural analysis of the molecular interactions between flavonoids and amino acids at the active site of CYP2D6 protein (Accession number: 3QM4) [17]. The existing ligand Prinomastat was used as the center for determining the active pocket of the CYP2D6 protein. Notably, only strong molecular interactions between flavonoids and the essential key amino acids (interaction energy<-1.5 kcal mol^{-1}) were highlighted in the 5 Å range of small ligands. The isoflavone Daidzein and its transformed flavonoid molecules were applied as an illustrational example for comparing different core flavonoid skeletons in the number of strong molecular interactions.

Structural similarity analysis

The SMILES strings of the 43 flavonoids and their corresponding transformed compounds were imported into Cytoscape 2.8.2 [18]. Each node represented a compound and different node colors were applied in order to distinguish between different categories. By selecting the minimum Taminoto value at 0.60, 0.65 and 0.70 successively, the plugin ChemViz was used to calculate the Taminoto coefficient defined structural similarity of every two compounds. If the calculated

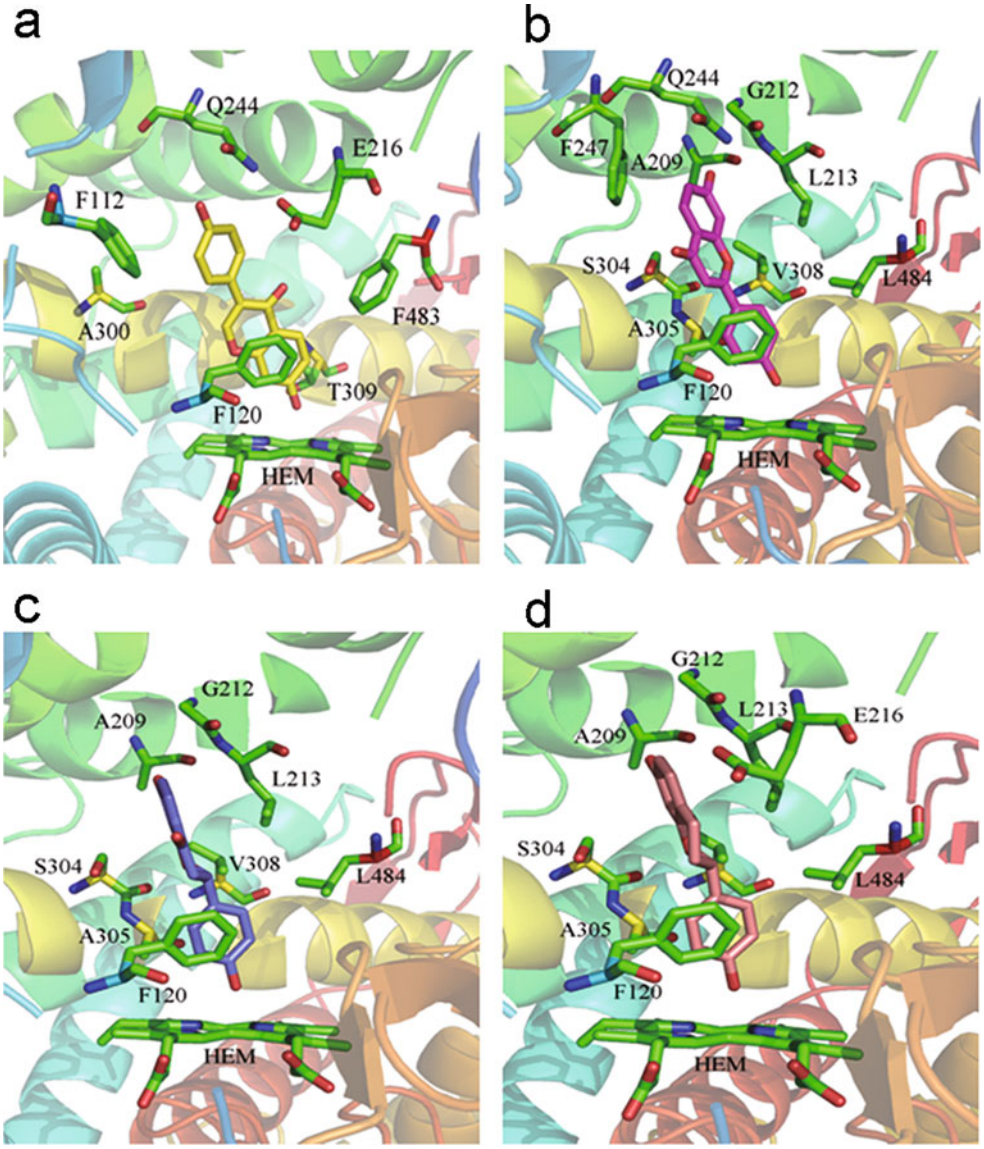

Fig. 2 Results of molecular interaction analysis. a-d. Models of Daidzein **a** and its transformed flavonoid molecules **b** C1 skeleton, **c** C2 skeleton, **d** C4 skeleton) binding to CYP2D6. Strong interactions with amino acids (interaction energy<-1.5 kcal mol^{-1}) were highlighted in the active pocket of CYP2D6. HEM: protoporphyrin IX containing Fe

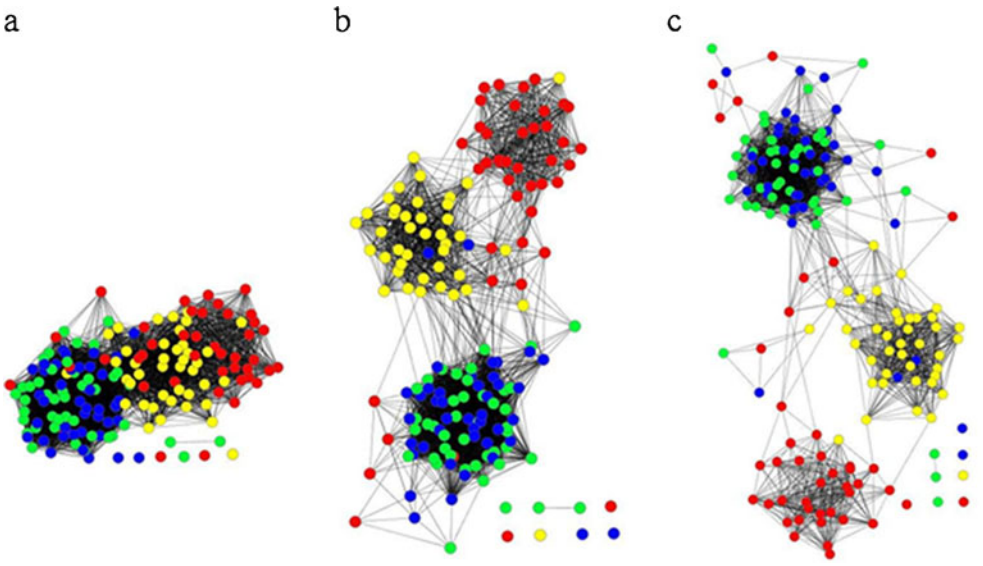

Fig. 3 Results of structural similarity analysis. **a-c** Structural similarity networks as the minimum taminoto value was set at 0.60, 0.65 and 0.70, respectively. Yellow nodes represent compounds in category 1 (flavones and flavonols); Blue nodes represent compounds in category 2 (flavanones and dihydroflavonols); Red and green nodes represent compounds in category 3 (isoflavones) and category 4 (flavans and catechins), respectively

Taminoto coefficient between two compounds exceeded threshold, the nodes representing compounds would be connected by edge. Finally, three structural similarity networks were created.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) for comparison among experimental groups or flavonoid categories. A P value of 0.05 was considered statistically significant and results were presented as mean ± SEM (standard error of mean). Comparison between two experimental groups was performed using Student's t test. GraphPad Prism v5.0 was used to perform the one-way ANOVA test.

Results and discussion

Isoflavones less likely inhibit CYP2D6 compared to other categories of flavonoids. In the current study, AutoDock 4.2 was applied to quantitatively evaluate the CYP2D6 inhibition extent of the flavonoids. In total 119 chemical compounds underwent virtual docking to the CYP2D6 protein (Supplementary data). After dividing the 119 compounds into two groups according to their experimentally validated Ki values, we developed a ROC prediction model of CYP2D6 inhibitor in the clinical context [14]. Area under the ROC curve (AUC) was 0.799 ($p<0.0001$, Fig. 1a). The cut-off value of docked Ki was 6.88 μM. The overall prediction accuracy was 74.8 %. This meant that our method could successfully distinguish potential CYP2D6 inhibitors from CYP2D6 non-inhibitors. The premise was that the docked Ki value of small molecules with CYP2D6 were known. Simply, if a compound was assigned with a docked Ki less than the cut-off value, it can be supposed that it would likely be a potent CYP2D6 inhibitor and cause unfavorable drug-drug interactions.

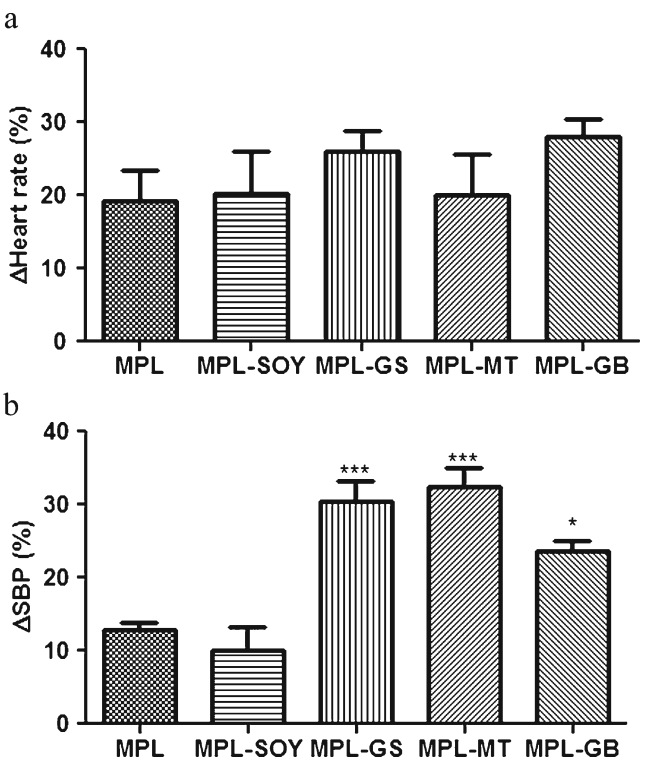

Fig. 4 Results of heart rate and systolic blood pressure measurement. **a** Percent decline of heart rate. **b** Percent decline of SBP. *$p<0.05$ or ***$p<0.001$ versus MPL group (n=5). MPL: metoprolol treated; MPL-SOY: metoprolol and soy extract co-treated; MPL-GS: metoprolol and grape seed extract co-treated; MPL-MT: metoprolol and milk thistle extract co-treated; MPL-GB: metoprolol and ginkgo biloba extract co-treated

Except ginkgo biloba extract, most flavonoid extracts mainly contain only one category of flavonoids, such as soy extract mainly containing isoflavones, while grape seed extracts mainly contain flavans and catechins. Based upon this fact, we proposed the hypothesis that flavonoid skeleton might contribute to the inhibitory differences of flavonoid extracts on CYP2D6 [9–13]. In order to prove our hypothesis, we picked up 43 typical flavonoids, which belonged to different categories of flavonoids (Supplementary data). After skeleton transforming (See Methods, Daidzein as an example shown in Fig. 1b) and virtual docking, we found that the inhibitory difference of flavonoids on CYP2D6 in greater extent dependent on the core skeletons instead of their substituents (Fig. 1c). The mean docked Ki value of isoflavones was significantly lower, compared to those of the other three categories of flavonoids, implying their weakest inhibition potential upon CYP2D6 ($p<0.05$, Fig. 1c). Furthermore, we performed a structural analysis to investigate the molecular interactions between flavonoids and the essential key amino acids at the active site of CYP2D6 protein. Although Schrodinger instead of Autodock and different crystal protein were used, the same result was obtained. Schrodinger assigned the smallest binding energy score to Daidzein, an isoflavone compound. Especially, The least number of strong interactions between Daidzein and amino acids (interaction energy<-1.5 kcal mol^{-1}) were found and structural transformation produced more strong molecular interactions (Fig. 2). Because any substituent was unchanged during structural transformation and only the core skeleton was altered, this result implied that the core skeleton of isoflavone would be responsible for the lowest binding affinity of Daidzein to CYP2D6.

In addition, we did not find clustering of each flavonoid with their respective transformed partners as the threshold of Taminoto coefficient increased (Fig. 3). The majority of compounds clustered according to their core skeleton, for example, isoflavones would cluster together (Fig. 3c). These results might consistently suggest that a flavonoid extract that mainly contained isoflavones would be more likely

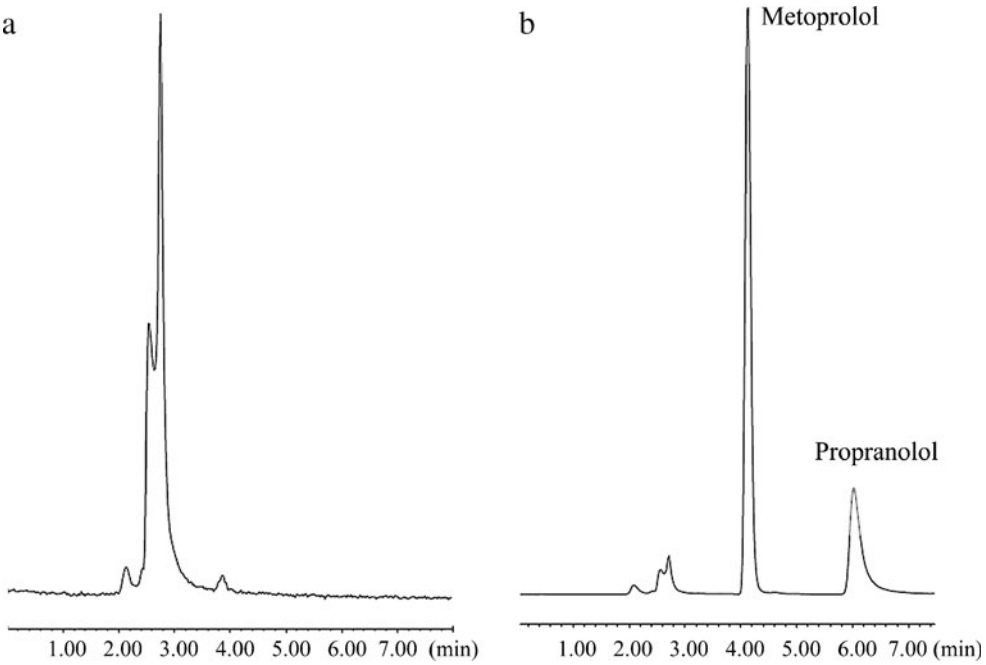

Fig. 5 Typical HPLC chromatograms. **a** Blank plasma sample. **b** Experimental plasma sample with internal standard propranolol

risk-free of the CYP2D6-mediated herb-drug interaction. The weak CYP2D6 inhibition shown by soy extract (the half maximal inhibitory concentration IC50>50 μM) has well verified this point [9]. On the contrary, grape seed extract was experimentally verified as a moderate CYP2D6 inhibitor [10] and CYP2D6 activity could be inhibited by milk thistle extract [11, 12]. By containing more than one categories of flavonoids (categories 1 and 4, See Methods), ginkgo biloba extract was proved decreasing the activity of CYP2D6 at low concentrations [13].

Co-administration of soy extract mainly containing isoflavones did not affect the PK/PD of metoprolol. Four typical flavonoid extracts were chosen for *in vivo* evaluation of the *in sillico* prediction results. The decline of systolic blood pressure (SBP) induced by metoprolol was significantly influenced by all the extracts except soy extracts (Fig. 4a). However, the heart rate response to metoprolol was not affected by all the co-administered flavonoid extracts (Fig. 4b). This was not consistent with the results obtained in the previous study [6]. It was doubted that the flavonoid extract co-administered buffered the role of increased concentration of metoprolol because cardiovascular ion channels were identified as molecular targets of flavonoids [19]. However, administration of flavonoid exact alone did not cause significant change in SBP and heart rate of rats (data not shown). Further experiments are needed.

As a long-term treatment was definitely needed for patients with IHD, we designed a 7 day experimental scheme of co-administration of metoprolol and each flavonoid extract to rats. The developed high-performance liquid chromatography (HPLC) method was applied to assess the impact of co-administration of flavonoid extracts on plasma concentration of metoprolol within a range from 0.05 to 10 μg/ml (Fig. 5). It was surprising that significant

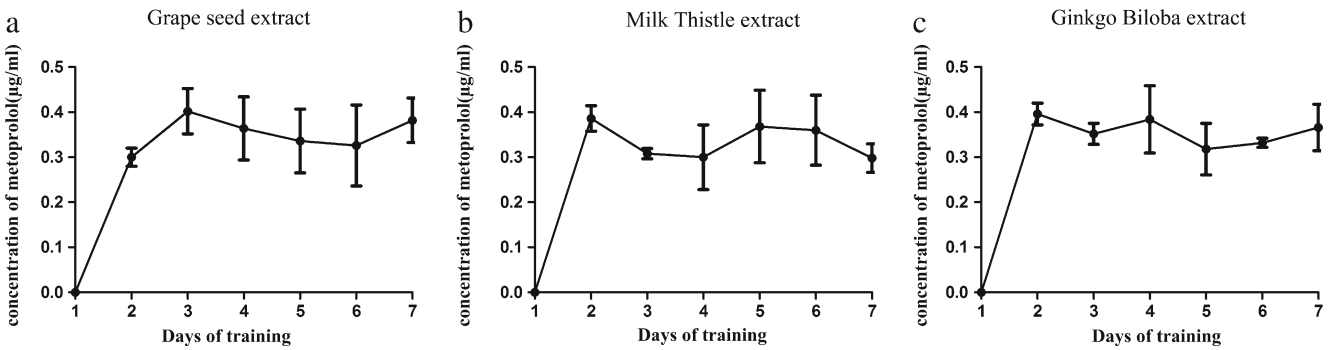

Fig. 6 Determination of metoprolol in rat plasma. **a-c** Impact of co-administered grape seed, milk thistle or ginkgo biloba extract on plasma concentration of metoprolol (n=5). MPL-GS: metoprolol and grape seed extract co-treated; MPL-MT: metoprolol and milk thistle extract co-treated; MPL-GB: metoprolol and ginkgo biloba extract co-treated

inhibition of metoprolol metabolism could be found in the MPL-GS, MPL-GB and MPL-MT groups just from day 2 (Fig. 6). However, no altered plasma concentrations of metoprolol in the MPL and MPL-SOY groups were detected by HPLC during the 7 days (data not shown), it might be on one hand because co-administration of soy extract did not affect the CYP2D6 activity and the metabolism of metoprolol and on the other hand due to contribution of the first-pass effect of metoprolol in rats [20].

Conclusions

Our results illustrated the significant differences of the most common four categories of flavonoids in herb-drug interactions, implying that rational selection of commercial flavonoid extracts was definitely needed for clinical application in IHD protection. Based upon our *in sillico* and *in vivo* evaluation results, herbal extracts rich in isoflavones are more appropriate to be developed as novel cardiovascular drug compared to other flavonoid extracts, due to their equivalent biological effects and lower risk of CYP2D6-mediated herb-drug interactions. Certainly, further experimental validation is definitely needed to detect our hypothesis.

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