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Original article

Interaction of quercetin and its metabolites with warfarin: Displacement of warfarin from serum albumin and inhibition of CYP2C9 enzyme

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ABSTRACT

Flavonoids are ubiquitous molecules in nature with manifold pharmacological effects. Flavonoids interact with several proteins, and thus potentially interfere with the pharmacokinetics of various drugs. Though much is known about the protein binding characteristics of flavonoid aglycones, the behaviour of their metabolites, which are extensively formed in the human body has received little attention. In this study, the interactions of the flavonoid aglycone quercetin and its main metabolites with the albumin binding of the oral anticoagulant warfarin were investigated by fluorescence spectroscopy and ultrafiltration. Furthermore, the inhibitory effects of these flavonoids on CYP2C9 enzyme were tested because the metabolic elimination of warfarin is catalysed principally by this enzyme. Herein, we demonstrate that each tested flavonoid metabolite can bind to human serum albumin (HSA) with high affinity, some with similar or even higher affinity than quercetin itself. Quercetin metabolites are able to strongly displace warfarin from HSA suggesting that high quercetin doses can strongly interfere with warfarin therapy. On the other hand, tested flavonoids showed no or weaker inhibition of CYP2C9 compared to warfarin, making it very unlikely that quercetin or its metabolites can significantly inhibit the CYP2C9-mediated inactivation of warfarin.

1. Introduction

Flavonoids are ubiquitous molecules in the nature. They are able to interact with many proteins (e.g., enzymes, transporters) in the human organism leading to their manifold biochemical and pharmacological effects in the body [1,2]. Flavonoids occur in several foods, drinks, herbal products, dietary supplements as well as some medications [2,3]. After oral consumption of flavonoid-containing foods or products, flavonoid aglycones undergo extensive presystemic elimination, resulting in the relatively low oral bioavailability of these compounds [4]. The rest of the parent compound and/or its metabolites can then reach the systemic circulation. Therefore the plasma concentration of the orally consumed or administered flavonoid aglycones is relatively low. Most of the flavonoids can bind to serum albumin with high affinity resulting in their strong albumin binding property [5–7].

Quercetin (Q) is one of the most commonly occurring flavonoid in the nature, it is contained by many fruits, vegetables, and grains [8,9]. Therefore, Q is part of the normal diet; furthermore, there are several dietary supplements containing high doses of Q (even 500–1000 mg in a tablet). During the normal diet, human plasma concentrations of Q and its metabolites are in the nanomolar range [9], while continuous supplementation with high doses of Q (500–1000 mg) can result in few micromolar levels in the circulation [10,11]. Like other flavonoid aglycones, Q is also highly metabolised [9,12]. Methylation of Q by catechol-*O*-methyltransferase (COMT) leads to the formation of isorhamnetin (IR; 3′-*O*-methylquercetin) and tamarixetin (TAM; 4′-*O*-methylquercetin); as COMT prefers more the 3′-*O*-methylation vs. 4′-*O*-methylation, considerably higher amounts of IR are formed [13–15]. Furthermore, sulfate and glucuronide conjugates of Q also appear [9,12];

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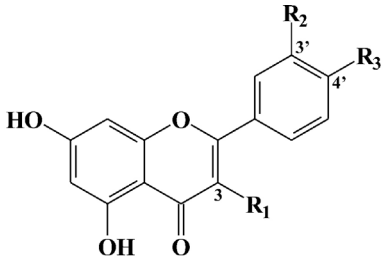
the dominant circulating metabolites of Q are quercetin-3′-sulfate (Q3′S), quercetin-3-glucuronide (Q3G), and isorhamnetin-3-glucuronide (I3G) [16]. Q binds to human serum albumin (HSA) with high affinity [6]. Recent in vitro studies demonstrate that Q is able to effectively displace strongly albumin-bound molecules from HSA causing the disruption of their albumin binding [17,18]. Previous investigations also suggest that some Q metabolites can bind to HSA with lower, similar or even higher affinity compared to the parent compound [19]; however, displacing abilities of the most important circulating Q metabolites (Q3′S, Q3G, and I3G) have not been reported.

Warfarin (WAR) is an orally-administered anticoagulant drug which is commonly used to prevent thrombosis and thromboembolism [20]. Because WAR is a drug with a narrow therapeutic window, the interaction of WAR with other drugs or dietary supplements can result in thrombus formation or bleedings in WAR-treated patients [21,22]. Therefore, the pharmacokinetic interactions of WAR are of high pharmacological as well as toxicological importance. More than 99% of WAR in the human circulation is albumin-bound form [23,24]. Thus, the displacement of only a few percent of WAR from HSA can dramatically increase the free concentration of WAR in the circulation. Previous in vitro studies demonstrated the strong displacing ability of flavonoids vs. WAR [18,25]. The main process responsible for the elimination of WAR is its biotransformation by CYP2C9 enzyme [26]. If the free concentration of WAR increases in the blood, and consequently in the liver cells, it is plausible to hypothesize that the metabolic inactivation of WAR by CYP2C9 becomes elevated as well, which can partly compensate the increased pharmacological effects of WAR. On the other hand, it was previously reported that the displacement of WAR from HSA by other drugs can cause bleeding [21,27]. These observations suggest that the strong displacement of WAR from albumin can result in serious consequences despite of the compensatory effects in the body. Furthermore, Q and IR are able to competitively inhibit the CYP2C9 enzyme [28,29], and the inhibition of CYP2C9-mediated metabolic inactivation of WAR by flavonoids can further aggravate the toxic consequences of the increased free (not HSA-bound) concentration of WAR.

In this study, we examined the albumin binding abilities of Q and its metabolites (Q3′S, Q3G, I3G, IR, and TAM; see in Fig. 1) as well as the potential inhibitory effects of these compounds on CYP2C9 enzyme. After the binding constants of flavonoid-HSA complexes were quantified by fluorescence quenching method, displacing abilities of Q and Q metabolites vs. WAR were demonstrated using fluorescence spectroscopic and ultrafiltration techniques. The effects of flavonoids on CYP2C9-catalysed hydroxylation of diclofenac were evaluated, and compared to the influence of the same amounts of WAR.



	R1	R2	R3
Quercetin (Q)	-OH	-OH	-OH
Isorhamnetin (IR)	-OH	-OCH_3	-OH
Tamarixetin (TAM)	-OH	-OH	-OCH_3
Quercetin-3'-sulfate (Q3'S)	-OH	-OH	-sulfate
Quercetin-3-glucuronide (Q3G)	-glucuronic acid	-OH	-OH
Isorhamnetin-3-glucuronide (I3G)	-glucuronic acid	-OCH_3	-OH

Fig. 1. Chemical structure of quercetin and its metabolites.

2. Materials and methods

2.1. Reagents

All reagents were of spectroscopic or analytical grade. Quercetin (Q) was purchased from Fluka. Isorhamnetin (IR) and tamarixetin (TAM) were purchased from Extrasynthese. Quercetin-3′-sulfate (Q3′S), quercetin-3-glucuronide (Q3G), and isorhamnetin-3-glucuronide (I3G) were synthetized as described previously [30]. Warfarin (WAR), human serum albumin (HSA) and CypExpress™ 2C9 (Cytochrome P450 human) kit were purchased from Sigma. Diclofenac (free acid) and 4′-hydroxydiclofenac were purchased from Carbosynth.

2.2. Fluorescence spectroscopic measurements

Fluorescence measurements were carried out employing Hitachi F-4500 fluorescence spectrophotometer. All measurements were performed at 25 °C. In order to mimic extracellular physiological conditions, albumin-ligand interactions and competitive interaction of flavonoids with WAR were investigated in phosphate buffered saline (PBS; pH 7.4).

Binding constants of Q and its metabolites with HSA were quantified with fluorescence quenching experiments. Fluorescence emission spectra of 2 μM HSA in the absence and presence of increasing flavonoid concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 μM) were recorded. Assuming 1:1 stoichiometry, binding constants were calculated by non-linear fitting with Hyperquad2006 (Protonic Software) using 295 nm and 340 nm as excitation and emission wavelengths, respectively [31,32]:

$$I = I_0 + \frac{(I_{HG} - I_0)}{2 \cdot [H]_0} \cdot \left([H]_0 + [G]_0 + \frac{1}{K} - \sqrt{\left([H]_0 + [G]_0 + \frac{1}{K} \right)^2 - 4 \cdot [H]_0 \cdot [G]_0} \right) \quad (1)$$

where I and I_0 denote the fluorescence emission intensity of HSA with and without flavonoids, respectively; I_{HG} is the fluorescence emission intensity of pure flavonoid-HSA complex (calculated by the Hyperquad2006); K denotes the binding constant (with the unit of dm^3/mol); while $[H]_0$ and $[G]_0$ are the total concentrations of HSA and flavonoids, respectively.

Evaluation of spectroscopic data with the graphical application of Stern-Volmer equation was also performed:

$$\frac{I_0}{I} = 1 + K_{SV} * [Q] \quad (2)$$

where I_0 and I are fluorescence emission intensities of HSA in the absence and presence of flavonoids, respectively. K_{SV} is the Stern-Volmer quenching constant while $[Q]$ is the concentration of the quencher.

To investigate the displacement of WAR from HSA by flavonoids, our previously described method was applied [31,33]. The complex formation of WAR with HSA results in the strong increase of its fluorescence (λ_{exc} = 317 nm, λ_{em} = 379 nm), therefore the complex formation as well as the displacement of WAR from HSA can be precisely followed. The increasing concentrations of HSA result in the gradual elevation of the fluorescence signal of WAR at 379 nm (Fig. S1) thus the concentration of the free and HSA-bound WAR can be calculated, as described previously [18]. In the presence of 1 μM WAR and 3.5 μM HSA, approximately 70% of the WAR molecules are presented in albumin-bound form. To achieve a