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Molecular displacement of warfarin from human serum albumin by flavonoid aglycones

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ABSTRACT

The well-known 4-hydroxycoumarin derivative warfarin is a widespread anticoagulant drug. Besides its strong albumin binding property warfarin has a narrow therapeutic window. Therefore, a few percent of displacement from albumin can result in serious biological consequences. The flavonoid molecular group also shows very strong plasma albumin binding characteristics occupying the same binding site. It is plausible to hypothesize that flavonoid aglycones may be able to displace warfarin from human serum albumin (HSA). In our study the competing activities of different flavone (acacetin, apigenin, chrysin, luteolin), flavonol (galangin, quercetin) and flavanone (hesperetin, naringenin) aglycones were investigated using fluorescence spectroscopy. Our results represent that flavonoids are able to displace warfarin from the surface of HSA. On the other hand, when comparing flavone or flavonol groups to flavanones the latter group seems to be much weaker competitor. These observations were also supported by calculation of stability constants. Our investigations strongly suggest that we should reckon with the described molecular displacement. However, further *in vivo* studies are needed to support the findings of our model system.

1. Introduction

The 4-hydroxycoumarin derivative warfarin (WAR, Fig. 1) is a widespread anticoagulant medication, it has been the primary anticoagulant drug used in the USA for more than 50 years [1]. It binds with a high affinity to plasma albumin [2,3]: about 99% of the circulating warfarin is bound [4]. Because of the high plasma protein binding only 1% displacement of warfarin can lead to doubling the free concentration of the drug in the blood. This property is coupled with a narrow therapeutic window therefore displacing of warfarin from human serum albumin (HSA) can result in the occurrence of unexpected bleedings. Because of the delicate balance of bound and unbound forms of warfarin in the human organism its molecular interactions is of very high importance [5,6]. Metabolism of warfarin is mainly driven by Cytochrome P450 (CYP450) enzymes (for example CYP2C9) [7].

Flavonoids are natural polyphenols which besides their scavenging activity have other important physiological effects as well *e.g.* strong plasma protein binding with competing ability [8] or inhibition of different enzymes and transporters [9]. Therefore this molecular group has become the focus of numerous biological researches. Interaction between warfarin and flavonoids seems to be a very interesting and complex problem because several flavonoids can inhibit CYP450 enzymes (like CYP2C9) [10–12] which may lead to decrease the metabolism of the drug. Furthermore in case of a few flavonoid derivatives anticoagulant and antiplatelet activity were also described [13,14], in addition some flavonoids could be able to displace warfarin from the surface of HSA [15] because the primary binding site of warfarin is located in subdomain IIA on HSA and previous studies concluded that flavonoid aglycones occupy the same binding site [2,15–17]. Numerous studies highlighted the possibility of drug-herbal interaction in case of warfarin [18–20]. Paeng et al. [21] and Hamann et al. [22] suggested that the flavonoid rich cranberry juice can interfere with warfarin therapy. In contrast with these observations, the meta-analyses done by Zikria et al. [23] did not show evidence to verify this effect. However, recent studies highlighted that the flavonol quercetin is susceptible to displace warfarin from

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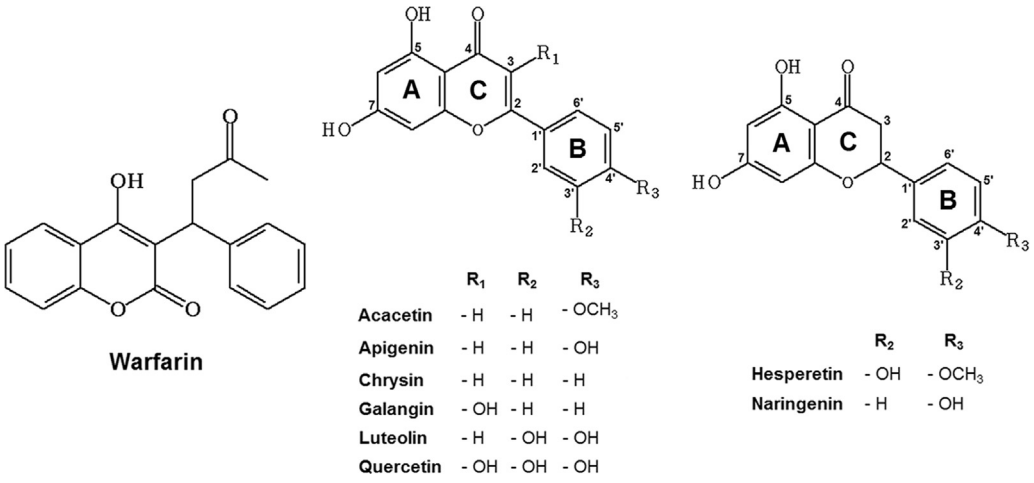

Fig. 1. Chemical structure of warfarin and the studied flavones (acacetin, apigenin, chrysin, luteolin), flavonols (galangin, quercetin) and flavanones (hesperetin, naringenin).

HSA [15,24]. In rat experiment designed by Chan et al. [25] the influence of rutin (a glycoside of quercetin) on the metabolism and therapeutic effect of warfarin was studied: elevation of free warfarin serum concentration and significant increase of the elimination half-life (S-enantiomer) were observed. Nevertheless, we still lack strong evidences regarding the biological feasibility of this interaction in case of expectable drug and polyphenol levels in human plasma. The therapeutic range of warfarin is about 2.5–5.0 μM (R- and S- configuration, respectively) in blood [4], however recent studies highlighted that quercetin concentration can reach several hundreds nM plasma levels (even 800 nM) [26–28]. In addition, nowadays there are numerous flavonoid-rich products which contain sometimes one pure glycoside or aglycone, in these cases plasma levels can achieve even the 1–1.5 μM concentrations [29]. Keeping in mind the nutrition-dependent potentially high quantity of circulating flavonoids our aim was to investigate the possible warfarin–flavonoid interaction.

Since warfarin has strong fluorescent property, fluorescence spectroscopy and steady-state fluorescence polarization techniques were applied to investigate warfarin–flavonoid interaction for albumin binding. Because there might be other possible pharmacokinetic and pharmacodynamic interactions between flavonoids and warfarin therefore, using an *in vitro* experimental model system is highly suitable to investigate the potential molecular displacement and its properties. For this reason we applied an *in vitro* warfarin–HSA complex system for investigation the displacement parameters in the presence of different flavonoid aglycons (in a quasi-physiological buffer). In our study, displacing capacity of 8 different aglycones (flavones, flavonols and flavanones) was examined (Fig. 1) using 3 μM warfarin and a wide range of flavonoid concentrations. This is the first time to investigate aglycones else than quercetin. Our examination explores some of the structural requirements of the displacing activity. Furthermore, combined effects of aglycone pairs were also tested. In addition competing effects of flavonoids were compared to 3 commonly used drugs. In order to support our results, the log$K$ values of different flavonoid aglycones were determined in the presence of warfarin.

2. Materials and methods

2.1. Reagents

Racemic warfarin (WAR), human serum albumin (HSA), galangin (GAL), naringenin (NAR), indometacin, furosemide, phenylbutazone (all from Sigma-Aldrich), acacetin (ACA), hesperetin (HES) (all from Extrasynthese), apigenin (API), chrysin (CHRY), quercetin (QUER) (all from Fluka), luteolin (LUT; from Indofine) were used as received. 2500 μM warfarin and 1000 μM flavonoid stock solutions were prepared in 96% ethanol (from Reanal, spectroscopic grade) and kept at 4 °C protected from light. Phosphate buffered saline (PBS) was used as a medium to mimic (extracellular) physiological conditions. PBS consisted of 137 mM NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4 and 1.5 mM K_2HPO_4 in tridistilled water (pH 7.4).

2.2. Instrumentation

Hitachi F-4500 fluorescence spectrophotometer and Fluorolog τ3 spectrofluorimetric system (Jobin–Yvon/SPEX) were used to obtain spectral properties and to determine steady state fluorescence polarization values of warfarin. All measurements were performed in the presence of air at +25 °C.

2.3. Data analyses

2.3.1. The degree of fluorescence polarization was calculated as

$$P = \frac{(I_{VV} - G \times I_{VH})}{(I_{VV} + G \times I_{VH})} \quad (1)$$

where I_{VV} and I_{VH} are intensities of vertically and horizontally polarized emissions respectively, and G is the actually measured instrument correction factor. Fluorescence polarization data were averaged from 15 measurement points.

2.3.2. To quantify the amount of albumin-bound warfarin the previously published calculations were applied [30]

$$c_b = \frac{[\Sigma I - a_f \times \Sigma c - (b_f + b_b)]}{(a_b - a_f)} \quad (2)$$

$$binding(\%) = \frac{100 \times c_b}{\Sigma c} \quad (3)$$

where ΣI is the measured intensity of the samples, Σc is the weighed warfarin concentration, c_f and c_b are the concentrations of free and bound warfarin, a_f and a_b are the slopes furthermore b_f and b_b are the intercepts of the calibration lines of the totally free and completely bound warfarin. OriginPro8 software (OriginLab Corp., Northampton, MA) was used for background correction