Inhibitory Effect of Hesperetin and Naringenin on Human UDP-Glucuronosyltransferase Enzymes: Implications for Herb–Drug Interactions

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Hesperetin (HET) and naringenin (NGR) are flavanones found in citrus (oranges and grapefruit) and *Aurantii Fructus Immaturus*. The present study aims to investigate the inhibition potential of HET and NGR derivatives towards one of the most important phase II drug-metabolizing enzymes—uridine diphosphate (UDP)-glucuronosyltransferases (UGTs). We used trifluoperazine as a probe substrate to test UGT1A4 activity, and recombinant UGT-catalyzed 4-methylumbelliferone glucuronidation was used as a probe reaction for other UGT isoforms. Data show that HET and NGR displayed broad-spectrum inhibition against human UGTs. Besides, HET exhibited strong inhibitory effects on UGT1A1, 1A3 and 1A9 (both IC50 and K1 values lower than 10µM), and the inhibitory effects of NGR against three major UGTs, including UGT1A1, 1A3 and 2B7. In a combination of inhibition parameters (Ki) and in vivo concentration of HET and NGR, the potential in vivo inhibition magnitude was predicted. Based on the reported maximum plasma concentration of HET and NGR in vivo, these findings indicate the potential herb–drug interactions (HDI) between HET or NGR and the drugs mainly undergoing UGT1A3 or UGT2B7 catalyzed metabolic elimination. Considering the variety of citrus that contains HET and NGR, so caution should be applied when taking drugs that utilize UGTs for metabolism and clearance with citrus fruits.

Key words herb–drug interaction; hesperetin; naringenin; uridine diphosphate (UDP)-glucuronosyltransferase

Regular Article

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Key words herb–drug interaction; hesperetin; naringenin; uridine diphosphate (UDP)-glucuronosyltransferase
activity have not been fully characterized. Understanding the effects of HET and NGR on UGT activities is important to ensure their safe administration and development new co-administration therapies with both drugs. The aim of this study was to investigate and compare the effects of HET and NGR on the activities of human UGTs. The potential for HDI in vivo was also quantitatively predicted and compared by using the area under the curve (AUC) ratios.

MATERIALS AND METHODS

Chemicals and Reagents  HET (purity: 98%) and NGR (purity: 98%) were purchased from Yi Fang Technology Co., Ltd. (Tianjin, China). Trifluoperazine hydrochloride (purity: 99.4%) and trifluoperazine-N-glucuronide were purchased from Toronto Research Chemicals (North York, ON, Canada). 4-Methylumbelliferone (4-MU), 4-methylumbelliferone-β-d-glucuronide (4-MUG), and 5′-diphospho-glucuronic acid trisodium salt (UDPGA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Isoimperatorin (purity: 99.6%), clarithromycin (purity: 99.4%) and magnesium chloride were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tris–HCl buffer (pH 7.4) was prepared by using Millipore filtered water (Milipore, Bedford, U.S.A.) and stored at 4°C until use. All other reagents were of the highest purity commercially available or HPLC grade.

Recombinant Human UGTs  Recombinant human UGT suprasomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in baculovirus-infected insect cells were obtained from BD Gentest Corp. (Woburn, MA, U.S.A.) and stored at −80°C until use.

Incubation of 4-MU Glucuronidation Assay  4-MU, a nonselective substrate of UGTs, was used as a probe substrate for all tested UGT isoforms (except trifluoperazine was used for UGT1A4). 4-MU was incubated with recombinant UGTs in the presence/absence of HET or NGR (1, 10, and 100 µM) in a reaction mixture (200µL) containing 50µM Tris–HCl buffer (pH 7.4), 5mm MgCl₂, and 3mm UDPGA. All components were dissolved in methanol and the final concentration of methanol in incubation system was no more than 0.5% (v/v). After a 5min pre-incubation at 37°C, UDPGA was added in the mixture to initiate the reaction. Incubation time and protein measurements were measured according to published methods with slight modifications details appear in Table 1. 4-MU and trifluoperazine concentrations corresponded to known Km or Km values for each enzyme. Reactions were quenched with adding 100µL ice-cold acetonitrile with isomperatorin (4µM) as an internal standard, then the mixture was placed on ice for 20min. Mixtures were vortexed for 5min and then centrifuged at 20000×g for 10min and then an aliquot of supernatant was transferred to an auto-injector vial for HPLC analysis. All experiments were performed in duplicate.

4-MUG concentrations were quantified using an HPLC system (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with four G1310A pumps, a G1329A auto injector, a G1341B VWD UV detector. Chromatographic separation was performed on a Luna C18 column (150×4.6mm, 5µm, Phenomenex) with the mobile phase consisted of acetonitrile (A) and 0.5% formic acid (B) at a flow rate of 1mL/min. The following gradient was used: 0–7min, 15–90% A; 7–9min, 90% A; 9–15min, 15% A. The column temperature was maintained at 25°C. The UV detector was set at 316nm. The total run time was 15.0min per sample for each metabolite, with a retention time for the peaks of 4-MUG (4.06min), 4-MU (5.58min), and isomperatorin (9.27min, internal standard). Peak shapes and separation efficiency for three compounds were achieved.

Incubation of Trifluoperazine Glucuronidation Assay  Trifluoperazine was used as a probe substrate for UGT1A4 because it does not conjugate 4-MU. Trifluoperazine hydrochloride was dissolved in H₂O and then incubated with recombinant UGT1A4 in presence/absence of HET or NGR (1, 10, and 100 µM) in a reaction mixture (200µL total volume). Then samples were treated and assayed as described above with clarithromycin (0.4µM) as an internal standard.

Trifluoperazine-N-glucuronide was analyzed on an LC/MS system (Shimadzu, Kyoto, Japan) equipped with UPLC and LCMS-2010EV instruments. Chromatographic separations were carried out using Luna C₁₈ column (50×2.0mm, 5µm) with a single quadrupole mass spectrometer. The mobile phase consisted of acetonitrile (A) and H₂O containing 5mmol/L ammonium (pH 3.7, B) (50:50, v/v) at a flow rate of 0.2mL/min. The column temperature was maintained at 25°C. MS parameters were: negative mode, curved desolvation line (CDL) temperature 250°C, heat block temperature 200°C, interface voltage 40V, detector voltage 1.3kV, and nebulizing gas (N₂) 1.5L/min. Quantification was performed with the selective ion monitoring mode of ions at m/z 584.52 for trifluoperazine-N-glucuronide and m/z 748.70 for clarithromycin (internal standard), and retention times were 1.26 and 1.21 min, respectively.

Kinetic Analyses for HET and NGR-Associated Inhibition on Recombinant Human UGTs  IC₅₀ values were obtained with various concentrations of HET and NGR. IC₅₀ values were calculated by non-linear regression analysis using Graphpad Prism 5.0. Glucuronidation velocity was measured using various concentrations of HET, NGR, and 4-MU or trifluoperazine (TFP). Kᵢ values were obtained using nonlinear regression and equations for competitive inhibition (Eq. 1), non-competitive inhibition (Eq. 2), or mixed inhibition (Eq. 3) as previously described. Actual experimental examples of uncompetitive inhibition are hard to find, so there is no equation

### Table 1. Incubation Conditions of 4-MU and Trifluoperazine Glucuronidation by Recombinant UGTs

<table>
<thead>
<tr>
<th>UGTs</th>
<th>Protein (mg/mL)</th>
<th>Incubation (min)</th>
<th>4-MU concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td>0.10</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>0.05</td>
<td>60</td>
<td>1000</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>0.10</td>
<td>20</td>
<td>TFP (40µM)</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>0.05</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>0.05</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>0.05</td>
<td>15</td>
<td>750</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>0.025</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>0.10</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>UGT2B4</td>
<td>0.20</td>
<td>120</td>
<td>1000</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>0.025</td>
<td>60</td>
<td>300</td>
</tr>
<tr>
<td>UGT2B15</td>
<td>0.10</td>
<td>30</td>
<td>250</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>0.20</td>
<td>120</td>
<td>2000</td>
</tr>
</tbody>
</table>
to show here. Dixon and Lineweaver plots (and plot slope) were used to confirm the inhibition type and calculate $K_i$ values.

$$v = \frac{V_{\text{max}}S}{K_m\left(1 + \frac{I}{K_i}\right) + S} \quad (1)$$

$$v = \frac{V_{\text{max}}S}{(K_m+S)\left(1 + \frac{I}{K_i}\right)} \quad (2)$$

$$v = \frac{V_{\text{max}}S}{(K_m+S)\left(1 + \frac{I}{aK_i}\right)} \quad (3)$$

Where $V$ is the velocity of the reaction; $S$ and $I$ are the substrate and inhibitor concentrations, respectively; $K_i$ is the inhibition constant describing the affinity of the inhibitor to the enzyme; $K_m$ is the substrate concentration at half of the maximum velocity ($V_{\text{max}}$) of the reaction. $K_i$ is the constant describing the affinity of enzyme-substrate complex for inhibitor.

**In Vitro—In Vivo Extrapolation (IV–IVE) to Predict in Vivo HET/NGR–Drug Interaction** The magnitudes of inhibitory interactions of HET and NGR were estimated as the equation of the area under the plasma concentration-time curve in the presence and absence of the inhibitor ($AUC_i/AUC$).

Ratios were calculated with the following Eq. 4:

$$\frac{AUC_i}{AUC} = \frac{1}{\left(1 + \frac{I}{K_i}\right)} + (1 - f_m) \quad (4)$$

Where $I$ is the concentration of inhibitor at the enzyme active site, and $f_m$ is the fraction of drug metabolized by the inhibited enzyme. $K_i$ is the inhibition constant describing the affinity of the inhibitor to the enzyme. When a drug’s clearance is solely mediated by a single enzyme ($f_m$=1), the $AUC_i/AUC$ equation can be simplified to Eq. 5 as previously utilized.20)

$$\frac{AUC_i}{AUC} = 1 + \left[I_{\text{in vivo}}/K_i\right] \quad (5)$$

Terms are defined as follows: $AUC_i/AUC$ is the predicted ratio of *in vivo* exposure of xenobiotics or endogenous substances with/without co-administration of HET or NGR. $K_i$ values are *in vitro* inhibition kinetic constants. $[I]_{\text{in vivo}}$ is the *in vivo* unbound concentration of HET or NGR. Ratios of $[I]_{\text{in vivo}}/K_i$ values were calculated to evaluate risks of UGT inhibition by HET and NGR. Based on the evaluation standard for HDI ($[I]_{\text{in vivo}}/K_i<0.1$, not possible; 0.1 $[I]_{\text{in vivo}}/K_i<1$, possible; $[I]_{\text{in vivo}}/K_i>1$, highly possible).23)

**RESULTS**

**Inhibition Screening of UGT Activities by HET and NGR in Recombinant Human UGTs** As seen in Fig. 2, the addition of HET (100 µM, final concentration) exhibited a strong or moderate inhibition on UGT1A1, 1A3, 1A4, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and the corresponding residual activities were 1.17, 3.56, 45.88, 23.71, 34.27, 6.74, 55.94, 51.78, 59.77, 59.73%, respectively. Likewise, as shown in Fig. 3, 100 µM of NGR exhibited a strong or moderate inhibition on UGT1A1, 1A3, 1A4, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and the corresponding residual activities were 3.25, 5.68, 38.72, 23.13, 29.71, 12.03, 38.55, 52.20, 3.71, 21.89%, respectively. Both HET and NGR had a negligible effect on the activities of UGT1A6 and 2B17. For UGT isoforms whose activity is inhibited by >50% by individual HET or NGR, IC$_{50}$ values of HET and NGR were further estimated.

To characterize inhibitory effects of HET and NGR towards UGT activities, dose-dependent inhibition curves were plotted with different HET and NGR concentrations and IC$_{50}$ data appear in Figs. 4A, 5A, 6A, 7A, 8A, 9A. Nilotinib (IC$_{50}$ for UGT1A1: 0.10 µM) and androstosterone (IC$_{50}$ for UGT2B7: 12.8 µM) were used as positive controls for inhibition assays.22,24) These results showed that HET strongly inhibited UGT1A1, UGT1A3, and UGT1A9 (IC$_{50}$<10 µM) and moderately inhibited UGT1A4, UGT1A7, UGT1A8 (IC$_{50}$ values 29.68–63.87 µM). NGR exhibited a strong inhibition on UGT1A1, UGT1A3, UGT2B7 (IC$_{50}$<10 µM) and a moderate inhibition on UGT1A4, UGT1A7, UGT1A8, UGT1A9, UGT1A10, and UGT2B15 (IC$_{50}$: 15–55 µM).

**Inhibition Kinetics of HET and NGR on Recombinant UGTs** Based on above IC$_{50}$ values (<10 µM), the inhibition types and inhibition parameters of HET on UGT1A1, 1A3,
Fig. 4. \( K_i \) of UGT1A1 by Hesperetin

Dose-dependent inhibition of HET against rhUGT1A1-catalyzed 4-MU glucuronidation and IC\(_{50}\) value (A), Dixon plot for HET inhibition of rhUGT1A1-catalyzed 4-MU glucuronidation (B), Lineweaver–Burk plot for HET inhibition of rhUGT1A1-catalyzed 4-MU glucuronidation (C), slopes from Lineweaver–Burk plot of HET inhibition of rhUGT1A1-catalyzed 4-MU glucuronidation (D).

Fig. 5. \( K_i \) of UGT1A3 by Hesperetin

Dose-dependent inhibition of HET against rhUGT1A1-catalyzed 4-MU glucuronidation and IC\(_{50}\) value (A), Dixon plot for HET inhibition of rhUGT1A3-catalyzed 4-MU glucuronidation (B), Lineweaver–Burk plot for HET inhibition of rhUGT1A3-catalyzed 4-MU glucuronidation (C), slopes from Lineweaver–Burk plot of HET inhibition of rhUGT1A3-catalyzed 4-MU glucuronidation (D).
Fig. 6. \( K_i \) of UGT1A9 by Hesperetin

Dose-dependent inhibition of HET against rhUGT1A9-catalyzed 4-MU glucuronidation and IC\textsubscript{50} value (A), Dixon plot for HET inhibition of rhUGT1A9-catalyzed 4-MU glucuronidation (B), Lineweaver–Burk plot for HET inhibition of rhUGT1A9-catalyzed 4-MU glucuronidation (C), slopes from Lineweaver–Burk plot of HET inhibition of rhUGT1A9-catalyzed 4-MU glucuronidation (D).

Fig. 7. \( K_i \) of UGT1A1 by Naringenin

Dose-dependent inhibition of NGR against rhUGT1A1-catalyzed 4-MU glucuronidation and IC\textsubscript{50} value (A), Dixon plot for NGR inhibition of rhUGT1A1-catalyzed 4-MU glucuronidation (B), Lineweaver–Burk plot for NGR inhibition of rhUGT1A1-catalyzed 4-MU glucuronidation (C), slopes from Lineweaver–Burk plot of NGR inhibition of rhUGT1A1-catalyzed 4-MU glucuronidation (D).
Fig. 8. $K_i$ of UGT1A3 by Naringenin

Dose-dependent inhibition of NGR against rhUGT1A3-catalyzed 4-MU glucuronidation and IC$_{50}$ value (A), Dixon plot for NGR inhibition of rhUGT1A3-catalyzed 4-MU glucuronidation (B), Lineweaver–Burk plot for NGR inhibition of rhUGT1A3-catalyzed 4-MU glucuronidation (C), slopes from Lineweaver–Burk plot of NGR inhibition of rhUGT1A3-catalyzed 4-MU glucuronidation (D).

Fig. 9. $K_i$ of UGT2B7 by Naringenin

Dose-dependent inhibition of NGR against rhUGT2B7-catalyzed 4-MU glucuronidation and IC$_{50}$ value (A), Dixon plot for NGR inhibition of rhUGT2B7-catalyzed 4-MU glucuronidation (B), Lineweaver–Burk plot for NGR inhibition of rhUGT2B7-catalyzed 4-MU glucuronidation (C), slopes from Lineweaver–Burk plot of NGR inhibition of rhUGT2B7-catalyzed 4-MU glucuronidation (D).
1A9 and NGR on UGT1A1, 1A3, 2B7 were investigated. The results (Figs. 4B & C, 5B & C, 6B & C) indicated that HET competitively inhibited UGT1A1-catalyzed 4-MU glucuronidation, and exerted mixed inhibition towards UGT1A3 and 1A9-catalyzed 4-MU glucuronidation. The \( K_i \) values were calculated to be 9.63, 0.99 and 3.41 \( \mu M \), respectively. Likewise, NGR was found to be a strong competitive inhibitor of UGT2B7 with a \( K_i \) of 1.39 \( \mu M \) (Figs. 9B & C). It also exerted intermediate non-competitive inhibition against UGT1A1 with \( K_i \) of 7.61 \( \mu M \) (Figs. 7B & C), as well as an intermediate uncompetitive inhibition against UGT1A3 with \( K_i \) of 85.76 \( \mu M \) (Figs. 8B & C).

### Data Extrapolation

**In vivo** extrapolation was used to generate pharmacokinetic data after ingestion of orange or grapefruit juice, and these data appear in Table 2. The results indicated that HET has demonstrated the potential risk of strong inhibition against UGT1A3, and NGR has shown the potential risk of inhibition towards UGT2B7.

### DISCUSSION

The present study investigated whether dietary flavonoids from citrus may cause herb–drug interactions. Our results demonstrated that both HET and NGR displayed broad-spectrum and strong or medium inhibition against human UGT isoforms except UGT1A6 and 2B17. Notably, 100 \( \mu M \) of HET exhibited more than 90% inhibition towards UGT1A1, 1A3, and 1A9. In contrast with HET, NGR had a strong inhibitory effect against UGT1A1, 1A3, and 2B7, and the residual activities were 4.395, 6.13, 6.04% at 100 \( \mu M \), respectively. Both of HET and NGR are showing moderate inhibitory effects against UGT1A7, UGT1A8. To understand the potential of HDI mediated by UGT enzymes, it is important to know the tissue distribution of these enzymes and the drug/metabolite concentrations in specific tissues. Moreover, UGT1A1, UGT1A3, UGT1A9, UGT2B7 are expressed in human liver. In contrast, UGT1A8 are expressed in the gastrointestinal tract, whereas UGT1A7 is present only in the esophagus, stomach, and lung. The HDI via inhibition of these UGTs may not only take place in human liver but also in the small intestine. UGTs in the gastrointestinal tract may contribute significantly to the first-pass metabolism of orally administered drugs that undergo glucuronidation. Our results showed that HET and NGR might affect the glucuronidation and first-pass metabolism of orally administered drugs.

Through the IV–IVE of HET and NGR, highly possible HDI between HET/NGR and drugs mainly undergoing UGT1A1/UGT2B7-catalysed metabolism might occur. It is well known that UGT1A3 and UGT2B7 are two major metabolizing enzymes responsible for the metabolic elimination of mitiglinide, a new potassium channel antagonist for the treatment of type 2 diabetes mellitus. 25) The likelihood of the interactions between HET or NGR containing herals and mitiglinide is very high when they are administered concomitantly. UGT1A3 efficiently catalyzes the glucuronidation of endogenous substances, such as estrogen and bile acids, and inhibition of UGT1A3 activity may disrupt the metabolism of these compounds. 26) UGT2B7 can also conjugate endogenous substances, including bile acids, androgens, and estrogens. 27) Many compounds have been reported to inhibit UGT2B7, such as arbidol and herbal andrographolide derivatives.28,29)

**In vitro** data tend to underestimate inhibition of drug glucuronidation in vivo. 30) Thus, HET and NGR might be more potent than data here suggest and individuals with greater systemic concentrations of HET and NGR may have increased the risk for herb–drug interactions, so IV–IVE should be done with care. 30) Also the inhibition behavior may be probe substrate-dependent and selection of different substrates changes the data regarding inhibition type and amount. 31) Moreover, complex herb constituents, processing methods, and environmental factors (soil, altitude, seasonal variation in temperature, length of daylight, rainfall pattern, shade etc.) may influence in vivo predictions. 32) Finally, individual differences in pharmacokinetics may change in vivo doses of HET or NGR and this may alter susceptibility to adverse effects with HET or NGR co-administration.

In summary, our finding shows that HET and NGR is a potent and broad-spectrum inhibitor against human UGT activity. Our prediction results for in vivo HDI demonstrate that the interaction is much likely to occur between high the dose of HET/NGR and the drugs which are substrates for the UGT1A3/UGT2B7. These data should be useful for guiding applications of orange juice or grapefruit juice, HET or NGR and related pharmaceutical preparations.

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### Conflict of Interest

The authors declare no conflict of interest.

### REFERENCES


