Glucuronidation of the red clover isoflavone irilone by liver microsomes from different species and human UDP-glucuronosyltransferases

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Abbreviations used: DAD, diode array detector; ESI, electrospray ionization; MUG, 4-(trifluoromethyl) umbelliferone-glucuronide; HFC, 7-hydroxy-4-(trifluoromethyl) coumarin; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; IF, isoflavone; IRI, irilone; IRI-G4´, IRI-O-4´-monoglucuronide; IRI-G5, IRI-O-5-monoglucuronide; MDP, methylenedioxyphenyl; MS, mass spectrometry; PLM, porcine liver microsomes; RLM, rat liver microsomes; UGT, UDP glucuronosyltransferase; UDPGA, uridine-5´-diphosphate-β,D-glucuronic acid ester
ABSTRACT

Red clover (Trifolium pratense L.) is used as a source for isoflavone (IF) dietary supplements. In this study we focused on the red clover IF irilone (IRI), because of its reported comparatively high bioavailability. Since the conjugative metabolism plays a key role in the elimination of IF, we investigated the species-specific differences and glucuronidation kinetics of IRI using different liver microsomes as well as the recombinant UDP-glucuronosyltransferases (UGTs) 1A1, 1A7, 1A8, 1A9, 1A10 and 2B15. Both possible monoglucuronides, the IRI-O-4’-monoglucuronide (IRI-G4’) and the IRI-O-5-monoglucuronide (IRI-G5), were detected. Human- (HLM) as well as rat liver microsomes (RLM) predominantly formed IRI-G5, whereas for porcine liver microsomes (PLM) IRI-G4’ prevailed. HLM showed an apparent V_max value of 0.43 nmol/min · mg and an apparent K_m value of 9.8 µM for the formation of IRI-G5 and a V_max of 0.35 nmol/min · mg and a K_m of 64.7 µM in case of IRI-G4’. Formation of both glucuronides was best fit using the substrate inhibition equation. The glucuronidation of IRI by UGTs led to values for the intrinsic clearance varying between 4 and 100 mL/min · mg with UGT1A7 showing the lowest and UGT1A10 the highest IRI conversion rate. The results indicate that IRI undergoes an efficient glucuronidation, presumably in the intestine and liver following atypical kinetic profiles.
INTRODUCTION

Isoflavones (IF) belong to the group of phytoestrogens displaying either weak estrogenic or antiestrogenic properties. Some, but not all epidemiological and clinical studies showed beneficial effects of IF on human health, such as maintaining bone density or alleviating menopausal symptoms in postmenopausal women (Jacobs et al., 2009; Liu et al., 2009). As a consequence, a growing market for dietary supplements based on soy and red clover as the major sources of IF has developed worldwide. However, in recent years there have been increasing concerns regarding the safety of such IF preparations, because animal studies provided evidence of potential adverse health effects which include an increased risk of breast cancer under certain conditions (Helferich et al., 2008).

Because the biotransformation seems to play a crucial role in the understanding and interpretation of the physiological effects of these compounds (Larkin et al., 2008; Lampe, 2009), a large number of studies investigated the metabolic pathways of IF in vitro and in vivo. To our knowledge all IF studied so far undergo, at least in part, a transformation by the colonic microbiota (Kelly et al., 1993; Heinonen et al., 2004; Ruefer et al., 2007). An exception is irilone (5,4′-dihydroxy-6,7-methylenedioxy-IF, IRI) which is almost resistant to microbial degradation (Braune et al., 2010). This might be seen as the major reason for the high bioavailability of IRI in comparison to other IF as very recently shown in a pilot intervention study (Maul and Kulling, 2010).

IRI possesses a methylenedioxy-(MDP) group attached to the aromatic A-ring of the IF skeleton which might impact its biological behaviour (Fig. 1). For example, other phytochemicals bearing a MDP-group, like safrol and piperin, are known to interfere with the
metabolism of drugs (Velpandian et al., 2001) and are described as inhibitors of cytochrome P450 enzymes (Ueng et al., 2005).

The conjugation of IF with activated glucuronic acid catalyzed by UDP-glucuronosyltransferases (UGTs) represents the major metabolic pathway for IF since more than 60% of the IF are renally excreted from the body in the monoglucuronide form (Adlercreutz et al., 1995). The aim of the present study was to characterize the UGT isoform specific glucuronidation of IRI in detail. Liver microsomes of three different species (human, rat and pig) were chosen as a common screening model. We hypothesized that the glucuronidation of each hydroxyl group of IRI is catalyzed by different UGTs (see Fig. 1). Therefore, human recombinant UGTs were applied in addition to the liver microsomes in order to investigate site-specific glucuronidation. Furthermore, the different expression levels of UGTs in various tissues permitted us to estimate in which tissue and to which extent glucuronidation might take place.

To date not much information about UGTs specificity for IF has been published and the few studies carried out so far indicate that a single isoform may convert two different IF to a totally different extent (Pritchett et al., 2008). For this study we selected those UGTs that so far been described to glucuronidate (iso)flavonoids (Lepine et al., 2004). For characterization of the glucuronidation patterns of IRI by various enzyme sources, incubations were carried out over a wide concentration range.
MATERIALS AND METHODS

Chemicals and enzymes

Irilone (9-hydroxy-7-(4-hydroxyphenyl)-[1,3]dioxolo[4,5-g]chromen-8-one, purity 98%, HPLC-DAD) was purchased from LGC Promochem (Wesel, Germany). 4-(Trifluoromethyl)umbelliferone-glucuronide (MUG) and UDP-glucuronic acid (UDPGA) were obtained from Sigma-Aldrich Chemical Co. (Deisenhofen, Germany). Irisolone (7-(4-hydroxyphenyl)-9-methoxy-[1,3]dioxolo[4,5-g]chromen-8-one) was isolated from the rhizome of Iris germanica (Weleda AG, Schwäbisch Gmünd, Germany). 7-Hydroxy-4-(trifluoromethyl) coumarin (HFC) and the human recombinant UGTs 1A1, 1A8, 1A9 and 2B15 were purchased from BD Bioscience (Heidelberg, Germany). Human recombinant UGTs 1A7 and 1A10 were obtained from Panvera/Invitrogen (Karlsruhe, Germany). Pooled human male and female liver microsomes (HLM) were from Advacell (Barcelona, Spain) and pooled liver microsomes from male Sprague Dawley rats were prepared according to standard procedures as described by Lake (Lake, 1987). PLM prepared from female pigs were a kindly provided by Prof. M. Metzler, Karlsruhe Institute of Technology, Germany. All chemicals were of the highest grade available.

Activity of microsomes and recombinant UGTs

The catalytic activity of the human recombinant UGTs used was measured according to the UGT Batch Data Sheet provided by the manufacturer. HFC was used as a reference substrate which is metabolized to MUG by the UGTs. MUG was quantified by HPLC-UV using an external calibration curve. To confirm the catalytic activity of the microsomes an analogous procedure was chosen (results are summarized in Supplemental Data Table 1).
Glucuronidation assay

The glucuronidation assay was carried out as described elsewhere (Matern et al., 1994; Fisher et al., 2000) with slight modifications. Human, rat and porcine liver microsomes or human recombinant supersomes were used. For a standard incubation, 0.075 mg supersomal and 0.028 to 0.058 mg microsomal protein were mixed with 5 µg alamethicin in 90 µL of 0.1 M potassium phosphate buffer (pH 7.4) and kept on ice for 10 min. MgCl₂ (final concentration 10 mM), the β-glucuronidase inhibitor D-saccharic acid 1,4-lactone (5 mM), and the substrate IRI dissolved in DMSO (final DMSO concentration 1.0 %) were added, and the mixture was preincubated at 37°C for 5 min. To initiate the reaction, UDPGA (final concentration 4 mM) was added resulting in a final volume of 200 µL and was then incubated for a further 90 min. The assays were terminated by adding 200 µL of 0.7 M glycine/HCl buffer pH 1.2. Alamethicin and the glucuronidase inhibitor were also added to the UGT experiments in order to assure comparable conditions in all incubations. All experiments were carried out in duplicate.

The incubation mixture was extracted with 2 x 800 µL ethyl acetate, the extract was evaporated to dryness, the residue dissolved in methanol/water (80/20; v/v) and analyzed by HPLC-DAD to measure IRI and its glucuronides. The substrate concentration for IRI ranged from 1.0 to 75 µM for determining kinetic data for the supersomal incubations and from 2.5 to 150 µM for the liver microsomal incubations.

HPLC-DAD-MS analysis

HPLC separation of IRI and its metabolites was carried out on a Prontosil (120 mm x 3.0 mm i.d., particle size 3 µm, pore size 120 Å) reversed-phase column (Bischoff, Leonberg, Germany). The solvent system consisted of 0.05% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was applied with a flow rate of 0.6 mL/min 0-50 min linear from 15%
to 35% B, 50-60 min linear from 35% to 40%, 60-63 min linear from 40% to 90% B followed by a reconditioning step for 5 min. 25 µL aliquots were used as standard injection volume. The analysis was performed on a HP 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with an auto injector, quaternary HPLC pump, column heater, DAD detector and HP Chem Station for data collection and handling. The eluent was recorded with a diode array detector at 270 nm. Observed peaks were scanned between 190 and 400 nm. The HPLC was interfaced to an HP series 1100 mass selective detector equipped with an API-ES chamber. The conditions for metabolite analysis in the negative electro spray ionization mode were: capillary voltage 3.5 kV; fragmentor voltage 80 V; nebulizing pressure 50 psi; drying gas temperature 350 °C; drying gas flow 12.5 L/min. Data were collected using the scan mode with spectra being scanned over a mass range of m/z 50 - 650.

Quantification of IRI was based on external calibration of the DAD signal at 270 nm. The detection limit, defined as three-fold base line noise, was 3.4 pmoles for IRI. Due to the lack of IRI monoglucuronides as reference substances for quantification, it was assumed that the monoglucuronides have molar extinction coefficients similar to that of their aglycone. This assumption was based on the observation that the sum of the peak areas of the IRI glucuronides and the remaining unconjugated IRI was comparable with the peak area for the IRI aglycone determined in the control incubation without the cofactor UDPGA. The areas obtained in both cases were almost identical. Furthermore, the recovery rate of IRI extracted from the control incubations with microsomal protein was almost identical to that of a matrix-free standard solution.

**Kinetic data analysis**
Each data point represents the mean of duplicate measurements. The apparent kinetic parameters $K_m$ and $V_{max}$ were calculated from the untransformed data by least-squares regression using GraFit 7.0.0 (Erithacus software limited, Horley, Surrey, UK). Data were fitted to the equations of the following kinetic models:

The Michaelis-Menten equation,

$$v = \frac{V_{max} \times S}{K_m + S}$$

where $v$ is the rate of reaction, $V_{max}$ is the maximum velocity, $K_m$ is the Michaelis Menten constant (substrate concentration at 0.5 $V_{max}$), and $S$ is the substrate concentration.

The Hill equation, which describes sigmoidal kinetics,

$$v = \frac{V_{max} \times S^n}{S_{50}^n + S^n}$$

where $S_{50}$ is the substrate concentration resulting in 50% of $V_{max}$ in Hill kinetic profiles and $n$ is the Hill coefficient.

The substrate inhibition model equation,

$$v = \frac{V_{max} \times S}{K_m + S \times (1 + \frac{S}{K_{si}})}$$

where $K_{si}$ is the constant describing the substrate inhibition interaction.

The two-site model equation,
\[
\frac{v}{V_{max}} = \frac{S}{K_s} + \frac{\beta S^2}{\alpha K_s^2} \\
1 + \frac{2S}{K_s} + \frac{S^2}{\alpha K_s}
\]

where \(K_s\) is the substrate dissociation constant and \(\alpha\) and \(\beta\) are binding factors that reflect changes in \(K_s\) and product formation (\(K_p\)), respectively (Houston and Kenworthy, 2000).

For reactions exhibiting Michaelis-Menten and substrate inhibited kinetics, intrinsic clearance (\(CL_{int}\)) was calculated as \(V_{max}/K_m\).

The goodness of the fit was determined by comparison of the statistical parameter \(\chi^2\) between the models as well as the standard errors of the various parameter estimates. The resulting data for the best fits are given in Table 1 as mean ± standard error of fit.
RESULTS

The in vitro glucuronidation of IRI was investigated using liver microsomes from three different mammalian species (human, rat, and pig) as well as six recombinant human UGTs. A representative HPLC chromatogram gave rise to two product peaks in addition to the IRI peak. The negative electrospray ionization (ESI) mass spectrum of the IRI peak exhibited a quasi molecular ions [M - H]− at m/z 297 whereas the ESI mass spectra of both product peaks had [M - H]− ions at m/z of 473 corresponding to IRI-O-monoglucuronides (Fig. 2). In addition, the IRI aglycon fragment ion at m/z 297 was found in the MS spectra of both product peaks resulting from the loss of the glucuronic acid moiety (176 Da). As IRI bears a hydroxyl function in position C-4′ as well as in position C-5, it can be concluded that both IRI glucuronides were formed (Fig. 1). No further peaks, additional to those that were also present in the control incubation could be detected. There was no indication for the formation of an IRI-diglucuronide.

A screening revealed that IRI is conjugated by all tested UGTs to at least one glucuronide, with the exception of UGT2B15, which led to no product formation under the incubation conditions used. At an IRI concentration of 25 µM, UGT1A1, UGT1A7 and UGT1A8 catalyzed the formation of both monoglucuronides while at a lower substrate concentration of 2.5 µM only one conjugate was detected. The UGTs 1A9 and 1A10 catalyzed the formation of one single monoglucuronide each independently of the IRI concentration. UGT1A9 gave rise to the product with the shorter retention time, whereas UGT1A10 exclusively formed the product eluting several minutes later in the RP-chromatography. This UGT selectivity was utilized for the structure elucidation of the two IRI monoglucuronides.

Structural considerations
A structural elucidation of the two IRI monoglucuronides was not feasible by NMR due to the low yield of glucuronide metabolites. In order to obtain the necessary structural information, glucuronidation of irisolone, the 5-methoxy-irilone derivative, with the UGTs 1A9 and 1A10 was carried out. In the case of irisolone, glucuronidation is possible only at the 4'-hydroxyl position. (Fig. 1). As expected, only one of the two UGTs, namely 1A10, metabolized irisolone while this IF was not converted by UGT1A9. In conclusion, solely UGT1A10 catalyzed glucuronidation of irisolone in position C-4'. We assume that UGT1A10 also catalyzed the C-4'-glucuronidation of IRI because of the high structural similarity. Thus, UGT1A9 converting IRI, but not irisolone, should be responsible for the glucuronidation in position C-5 which is not accessible in the irisolone structure. In the following sections, the monoglucuronides will be labelled according to this assumption with IRI-G5 for the glucuronide conjugated in position C-5 and IRI-G4' for the C-4' conjugate, respectively.

**Kinetic profiles of the IRI glucuronidation derived from microsomal experiments**

Incubation of IRI with each of the mammalian liver microsomes in the presence of UDPGA led to the formation of IRI-G5 and IRI-G4'. A significant difference in the pattern of the glucuronide formation could be observed depending on the mammalian species, as well as on the substrate concentration. Remarkably, in all initial microsomal incubations in which only two substrate concentrations were used, the intensity of the IRI-G4' formation increased more strongly with higher substrate concentrations when compared to the IRI-G5 formation indicating a kinetic progression. Therefore, IRI glucuronidation activities of the three mammalian liver microsomes were determined over a concentration range from 2.5 to 150 µM IRI (135 µM for HLM).

In Fig. 3 the results are presented as plots of the glucuronidation activity versus the IRI concentration for the formation of the two glucuronides. As expected from the initial
experiments, none of the plots showed typical Michaelis-Menten kinetics and thus did not allow a satisfactorily display of the kinetic data using Lineweaver-Burk plots. Additionally, results are presented as Eadie-Hofstee plots in order to examine the atypical kinetic behaviour by direct visual inspection (Miners et al., 2010). The formation of IRI-G4´ and IRI-G5 by HLM as well as of IRI-G5 by RLM exhibited substrate inhibited kinetics (Fig. 4 A, B and C). In contrast, the glucuronidation of IRI in position C-4´ catalyzed by RLM showed sigmoidal or autoactivation kinetics (Fig. 4 D). Formation of IRI-G5 by PLM showed a Michaelis-Menten kinetic profile with slight substrate inhibition characteristics. Unfortunately, the formation of the dominating product IRI-G4´ led to an Eadie-Hofstee plot that cannot be evaluated (Fig. 4 E and F). In summary, comparing the three mammalian species, significant differences were observed.

Beside the glucuronidation kinetics, also the activities for the formation of the two glucuronides varied among the different species. While HLM and RLM predominantly formed IRI-G5, PLM catalyzed the formation of IRI-G4´ to a much higher extent. This is reflected in the kinetic parameters obtained by fitting of the untransformed data to the equations of the four kinetic models. The calculated kinetic parameters for the model that provided the best fit according to least squares regression analysis are summarized in Table 1.

IRI-G5 formation followed substrate inhibited kinetics for the microsomes from all three species. The $V_{\text{max}}$ values were 1.37 nmol/min · mg for RLM, 0.43 nmol/min · mg for HLM and 0.28 nmol/min · mg for PLM. On the other hand, the $K_m$ value was higher for the PLM ($K_m = 16.8 \mu$M) than for the HLM ($K_m = 9.8 \mu$M), but still lower than the one for RLM ($K_m = 34.0 \mu$M).

The substrate inhibition decreased in the row RLM ($K_s = 65.3 \mu$M) being most strongly inhibited, followed by HLM ($K_s = 96.9 \mu$M) which was less inhibited to PLM which showed almost no inhibition ($K_{si} = 183.4 \mu$M). Only in case of RLM the formation of IRI-G5 fitted to the
two-site model equation was comparable regarding the goodness than fitting to the substrate inhibition equation. The following parameters were calculated: \(K_s, 91.5 \pm 11.4 \mu M; V_{\text{max}}, 3.46 \pm 0.33 \text{ nmol/min} \cdot \text{mg}, \alpha, 0.13 \pm 0.06; \beta, 0.06 \pm 0.02.\)

While HLM also catalyzed the IRI-G4´ formation in a substrate inhibition manner \((V_{\text{max}}, 0.34 \text{ nmol/min} \cdot \text{mg}; K_m, 64.7 \mu M \text{ and } K_{\text{si}} = 50.9 \mu M),\) the type of the kinetic profile could not be accurately estimated for RLM and PLM based either on the fitting of the untransformed data or the Eadie-Hofstee plots (Fig. 4D and 4F). For IRI-G4´ formation by RLM, the data were fitted to the Hill as well as to the substrate inhibition equation with the result of a similar goodness of fit \((\chi^2 \text{ of } 0.00037 \text{ and } 0.00016, \text{ respectively})\) in both cases. The additional fit according to the equation of the two-site model led to a slightly higher \(\chi^2\) value. For IRI-G4´ formation catalyzed by PLM all fits showed rather poor \(\chi^2\) values with the best one being 0.0023 for the substrate inhibited kinetics resulting in a very high \(V_{\text{max}}\) value of 4.87 nmol/min \cdot mg, a \(K_m\) of 88.6 \mu M and a \(K_{\text{si}}\) of 49.1 \mu M. The fit to substrate inhibited kinetics of IRI-G4´ formation by RLM resulted in the following parameters: \(V_{\text{max}}, 1.33 \text{ nmol/min} \cdot \text{mg}; K_m, 124.5 \mu M \text{ and } K_{\text{si}}, 44.7 \mu M.\)

An assumed auto-activated formation of IRI-G4´ by RLM showed a \(V_{\text{max}}\) of 0.294 nmol/min \cdot mg, a \(S_{50}\) value of 14.8 \mu M and a degree of sigmoidity with \(n = 2.1.\) Thus, remarkable differences between the species were observed, especially regarding the \(V_{\text{max}}\) values derived for the IRI-G4´ formation (Table 1).

**Kinetic profiles of the IRI glucuronidation derived from UGTs**

Six human recombinant UGTs (1A1, 1A7, 1A8, 1A9, 1A10 and 2B15) were selected, and tested for their ability to glucuronidate IRI. This screening showed that IRI was a suitable substrate for all the tested enzymes except for UGT2B15 which did not metabolize IRI. All tested members of the UGT1A subfamily metabolized IRI to at least one glucuronide. UGT1A10 only
led to the formation of IRI-G4´, while UGT1A9 and 1A7 solely formed IRI-G5 independent of the IRI concentration. In case of other UGTs the substrate concentration clearly influenced the formation of the glucuronide isomers: While at a rather low IRI concentration of 2.5 µM the UGTs 1A1 and 1A8 only the formation of IRI-G5 was observed, IRI-G4´ was also formed at a ten-fold higher substrate concentration.

For elucidating the activity of the UGT isoforms in a substrate concentration range from 1 µM to 75 µM, the kinetic profiles for the glucuronidation of IRI were assessed. For almost all UGTs, a Michaelis-Menten type kinetic was observed for concentrations below 20 µM. The enzymatic activity started to decrease at IRI concentrations higher than 20 µM indicating substrate inhibited kinetic profiles (Fig. 5). Thus, the evaluation of the kinetic parameters was carried out by fitting according to the different kinetic model equations described in Material and Methods.

All UGTs showed similar substrate inhibited kinetic profiles except for UGT1A9 which catalyzed the IRI-G5 formation with an almost Michaelis-Menten-like kinetics over the whole of the substrate concentration range. The apparent substrate inhibition constant Kₛᵢ was higher than 500 µM, and therefore almost negligible. The fitting of the untransformed data to the equation for the substrate inhibited kinetics for the formation of IRI-G5 by UGTs 1A1, 1A7 and 1A8, led to widely varying kinetic parameters. The highest Vₘₐₓ was observed for UGT1A8 which was almost thirty-fold higher than the value obtained for UGT1A7. Although UGT1A8 led to the formation of both glucurinides, IRI-G5 was dominantly formed and Vₘₐₓ was two-fold higher when compared to that of the IRI-G4´ formation.

UGT1A10 which exclusively formed IRI-G4´, did not allow a good fit for any of the kinetic models considered. However, the best fit expressed by the lowest χ² was obtained for the
substrate inhibited kinetics. Table 1 summarizes the kinetic parameters obtained by fitting of the various glucuronidation data.

Due to the weak product formation the conjugation in position C-4′ mediated by UGTs 1A1 and 1A7 could not be fitted satisfactorily to any of the equations. Hence, these data were excluded from the kinetic analysis.

**Intrinsic clearance**

The values for the intrinsic clearance (Cl\textsubscript{int}) represent the sum of the values for both IRI glucuronides. In almost all cases the goodness of fit for the substrate inhibition was at least gradually better than for the Hill kinetics. Therefore, only Cl\textsubscript{int} values are given. Considering the recombinant enzymes, IRI clearance by UGT1A10 exhibited the highest value (99.9 ± 46.5 mL/min · mg protein). Cl\textsubscript{int} of the other enzymes decreased in the following order: UGT1A9, 53.3 ± 16.6 mL/mg · min; UGT1A1, 27.2 ± 8.9 mL/mg · min; UGT1A8, 20.2 ± 3.9 mL/mg · min; UGT1A7, 3.9 ± 1.2 mL/mg · min. Thus, the catalytic efficacy of UGT1A10 was twenty-fold higher than the one measured for 1A7. The clearances found for the three mammalian microsomes were all in a comparable range with a Cl\textsubscript{int} 71.8 ± 24.4 mL/mg · min for PLM, 51.0 ± 17.4 mL/mg · min for RLM and 48.5 ± 10.4 mL/mg · min for HLM.
DISCUSSION

The formation of the two possible monoglucuronides, the C-4´ glucuronide and the C-5 glucuronide was documented by means of LC-M and tentatively assigned to the two new product peaks observed. This assignment was made based on the assumption that UGTs 1A9 and 1A10 show the same regioselectivity for IRI than for its monomethylated derivative irisolone.

All liver microsomes used in the study as well as the UGTs 1A1 and 1A8 catalyzed the formation of both glucuronides while the UGTs 1A7, 1A9 and 1A10 only gave rise to one site-selective product under the assay conditions employed in this study. UGT2B15 did not convert IRI to any glucuronide. The UGTs 1A7 and 1A9 led to the formation of the C-5 conjugate, whereas UGT1A10 exclusively formed the C-4´ glucuronide. As only a set of the six most important UGTs was studied, it cannot be ruled out that further UGTs are able to glucuronidate IRI. In particular the UGTs 1A3 and 2B7 should be investigated in future because they are known to metabolize estradiol (Lepine et al., 2004).

The microsomal glucuronidation of IRI in position C-5 was the dominating reaction for HLM and RLM, while the glucuronidation in position C-4´ prevailed for PLM. Although three of the tested recombinant human UGTs (1A1, 1A8 and 1A10) gave rise to the metabolite conjugated in position C-4´, quite low amounts of IRI-G4´ were detected after incubation with HLM. This may be explained by the fact that UGT1A10, as the main IRI-G4´ producing enzyme, represents an extrahepatic isoform not being expressed in human liver tissue (Tukey and Strassburg, 2000). The predominant occurrence of IRI-G5 correlates well with the fact that UGTs 1A9 and 1A1 are commonly expressed in the human liver (Tukey and Strassburg, 2000) both exclusively (UGT1A9) or at least predominantly (UGT1A1) forming the C-5 glucuronide. One might
conclude that in vivo the C-4´ glucuronidation occurs in considerable amounts in the intestine, where UGT1A10 is predominately expressed.

The regiospecificity of the IRI glucuronidation by UGTs is supported by the findings of Joseph et al. who examined the glucuronidation of the red clover IF prunetin (4´,5-dihydroxy-7-methoxy-isoflavone) (Joseph et al., 2007). As observed in our study for IRI, the UGTs 1A10, 1A8 and 1A1 (in the order of decreasing activity) were also mainly responsible for the 4´-glucuronidation of prunetin. The formation of the prunetin-5-O-glucuronide was catalyzed by UGTs 1A9, 1A8, 1A7 and 1A1, very similar to the results presented for IRI in this study. We assume that this specificity of the UGTs is characteristic for the glucuronidation of IF, bearing only two available hydroxyl functions in the positions C-4´ and C-5. However, this is no longer the case as soon as an additional hydroxyl group is present in position C-7 of the IF skeleton. This was demonstrated for genistein (4´,5,7-trihydroxy-isoflavone), where a dominant formation of the 4´- and 7-, but not of the 5-O-glucuronide, was observed (Doerge et al., 2000).

Kinetic analysis of the glucuronidation of IRI, including the intrinsic clearance estimates (CL_{int}), allows to draw some conclusions regarding the in vivo situation. In our glucuronidation experiments, a tendency towards a substrate inhibited kinetic profile was observed in almost all cases. However, some exceptions were observed: for the formation of IRI-G4´ by RLM but also by PLM, an auto-activated kinetic profile cannot be completely ruled out based on the goodness of the fit of the untransformed data and of the Eadie-Hofstee plots. Depending on the model chosen, the kinetic parameters calculated for the glucuronidation by microsomes can vary tremendously which is not observed in case of single recombinant UGTs. This can be explained by the fact that microsomes comprise a pool of UGT enzymes.

Moreover, UGT1A9 catalyzed the formation of IRI-G5 in an almost Michaelis-Menten-type kinetic with a tendency to a substrate inhibition. UGTs 1A1 and 1A8, both forming two
glucuronides, exerted a different kind of atypical kinetics: At low substrate concentrations, both isoforms did not catalyze an IRI-G4' formation whereas at concentrations higher than 2.5 µM in case of UGT1A8 and 7.5 µM in case of UGT1A1 the C-4' glucuronide could be detected. For UGT1A8 mathematical fitting gave evidence for substrate inhibited kinetics with a rather strong K_{si} and a weak K_{m} for both glucuronides. A similar tendency was observed for UGT1A1. This atypical kinetic cannot explain the delayed formation of IRI-G4'. Thus, for those enzymes giving rise to both products an interference of the formation of IRI-G4' caused by the conversion of IRI to IRI-G5 must be considered. One possible explanation may be a higher binding affinity for a particular binding pose of the IRI molecule into the active site of the specific UGT which results in the formation of IRI-G5. IRI-G4' is formed only at substrate concentrations close to saturation (or V_{max}) of the stronger binding site.

Furthermore, there is compelling evidence indicating that UGTs are oligomeric enzymes (Finel and Kurkela, 2008). Thus, atypical kinetic profiles could also result from interactions between the single monomer units in the (hetero)-oligomer. This might also lead to a modulation of the IRI-G4' formation caused by IRI-G5 formation in a second monomer of the complex.

When the IRI concentration exceeds ca. 20 µM, substrate inhibited kinetics could be observed in most cases. The IF concentration in blood plasma after ingestion of usual doses of dietary supplements does not reach this level (King and Bursill, 1998; Richelle et al., 2002). However, after intake of very high doses of IF, plasma levels higher than 15 µM were reported (Takimoto et al., 2003). The IF plasma concentrations according to the manufacturers’ recommended intake of red clover based dietary supplements are as high as 1 µM (Howes et al., 2002; Maul and Kulling, 2010) and, therefore, the formation of the C-5 glucuronide should be dominating in the liver. Formation of the IRI C-4' glucuronide by the UGTs investigated in this study can hardly be expected in the liver. However, UGT1A10 which mainly catalyzed the IRI-G4' formation, is
predominantly located in the small intestine. Therefore, glucuronidation in the intestinal epithelium directly after absorption might be possible.

It is noteworthy that the PLM seems to exhibit a different UGT activity pattern in comparison to HLM and RLM, as demonstrated by a dominantly C-4′ glucuronide formation. In contrast, the glucuronidation pattern obtained by incubations with RLM comes at least close to the results obtained for HLM. The finding that the glucuronidation pattern and kinetics are highly species dependent has already been described by Joseph et al. (2007) pointing out the differences between human, mouse and rat liver microsomes.

Generally, our data showed high values for the intrinsic clearance of IRI. Thus, it can be assumed that the compound will be rapidly conjugated after absorption. This conforms with previous results that show intense conjugative metabolism for the IF genistein and daidzein (Sfakianos et al., 1997; Doerge et al., 2000; Hendrich, 2002). Pritchett et al. (2008) investigated the conjugative metabolism of daidzein and genistein by a panel of human liver microsomes and found activities as high as 0.5 nmol/min · mg for UGT1A9 and 1A10 at 100 µM substrate concentration. Although kinetic parameters were not determined, the data for the highest conversion rates are very similar to our results for IRI. In contrast, Doerge et al. (2000) found an enzymatic activity for the conversion of the same IF, being about one order of magnitude lower. Regardless of the substrate it is generally difficult to compare enzyme kinetics as soon as no standard reference substrate is included to determine the basic activity of the enzymes used. For example, Soars et al. and Fisher et al. both present data for the glucuronidation of 17β-estradiol applying the same kinetic models but with rather deviating values for $V_{\text{max}}$ and $K_m/S_{50}$ (Fisher et al., 2000; Soars et al., 2003). This emphasizes the need to determine the UGT activity based on a commonly accepted reference substrate such as umbelliferone.
In conclusion, for the glucuronidation of IRI by almost all human UGTs it can be stated that similar to prunetin but in contrast to genistein, the C-5 position appears to be the preferred site of conjugation. With increasing substrate concentrations, the C-4´ conjugation gains more importance. The formation of both glucuronides can in all cases be best described according to substrate inhibited kinetics. Because a strong glucuronidation activity of IRI by UGT1A10 was observed, the glucuronide conjugation should occur already in small intestine. Here, the formation of IRI-G4´ as the conjugation product of UGT1A10 seems to be feasible. The in vitro values obtained for the intrinsic clearance of IRI by microsomes and UGTs in general are relatively high when compared to other xenobiotics. Thus, a rapid conversion could be expected to occur in vivo.
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AUTHORSHIP CONTRIBUTION

Participated in research design: Maul and Kulling.

Conducted experiments: Maul and Siegl.

Performed data analysis: Maul, Siegl and Kulling.

Wrote or contributed to the writing of the manuscript: Maul and Kulling.
REFERENCES


FIGURES

**Fig. 1:** Structural elucidation of the irilone glucuronides based on the UGT isoform selective glucuronidation observed: UGT1A9 and UGT1A10 both exclusively conjugate irilone to one distinct monoglucuronide either in the C-5- or the C-4’ position. Additionally irisolone is only metabolized by UGT1A10, thus leading to the conclusion that the UGT1A10 is responsible for the glucuronidation at C-5 in both isoflavone structures, irilone and irisolone.

**Fig. 2:** HPLC-ESI(-)-MS extracted ion chromatograms of the quasi molecular ion of irilone-O-monoglucuronide (m/z = 473) and irilone aglycone (m/z = 297) resulting from the incubation of irilone (50 µM) with RLM (0.5 mg protein/mL) for 90 min.

**Fig. 3:** Plots of the rate of product formation of irilone-5-O-glucuronidation (IRI-G5, plot A) and irilone-4’-O-glucuronidation (IRI-G4’, plot B) versus the substrate concentration by human liver microsomes (▲), rat liver microsomes (●) and porcine liver microsomes (♦). The points represent the experimentally determined IRI glucuronide formation rates at IRI concentrations in the range of 1 to 150 µM. Each data point represents the mean value of two independent measurements.

**Fig. 4:** Eadie-Hofstee plots of the irilone (IRI) glucuronidation by human liver microsomes (HLM), rat liver microsomes (RLM) and porcine liver microsomes (PLM). Plots A and B show the formation of IRI-G5 and IRI-G4’ by HLM. Plots C and D show the formation of IRI-G5 and IRI-G4’ by RLM, E and F are derived from the incubations with PLM, respectively. For IRI-G4’ formation by PLM the plot cannot be described by linear regression or a curve.
Fig. 5: Plotting of the product formation versus substrate concentration for the two glucuronides after incubation with UGT1A1 (♦), UGT1A7 (●), UGT1A8 (▲), UGT1A9 (■) and UGT1A10 (▼). Plot A depicts the fits for the formation of irilone glucuronide IRI-G5 and plot B for the formation of IRI-G4’. Each sample point represents the mean value of analyses carried out in duplicate.
**Table 1:** Apparent kinetic parameters for the glucuronidation of irilone (IRI) by various UGT enzymes and mammalian microsomes calculated based on fitting to the substrate inhibition equation as described in Material and Methods. Fitting to the Hill equation was only possible in case of the formation of IRI-G4’ by RLM (V\(_{\text{max}}\) 0.29 ± 0.01 nmol*mg\(^{-1} \star\)min\(^{-1}\), S\(_{50}\) = 14.8 ± 1.5 µM, n = 2.1 ± 0.4; see explanation to Hill equation in Material and Methods)

<table>
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<tr>
<th>Protein Source</th>
<th>IRI-G5</th>
<th></th>
<th></th>
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<th>IRI-G4’</th>
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<tr>
<td></td>
<td>V(_{\text{max}})</td>
<td>Km</td>
<td>Ksi</td>
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<td>[nmol*mg(^{-1} \star)min(^{-1})]</td>
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<td>UGT1A 1</td>
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<td>8.5 ± 2.9</td>
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<td>UGT1A8</td>
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<td>UGT1A10</td>
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<td>ND</td>
<td>1.03 ± 0.23</td>
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<td>RLM</td>
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<td>PLM</td>
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<td>183.4 ± 49.9</td>
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<td>88.6 ± 26.0</td>
<td>49.1 ± 22.1</td>
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</table>

N.D. = glucuronide not detected; a = no fitting possible
Figure 1

irilone (IRI)

UGT 1A9

IRI-G5

no conjugation possible

UGT 1A10

R = H: IRI-G4'
R = CH₃: irisolone-G4'

irisolone
Figure 2

--- XIC m/z 473
--- XIC m/z 297

intensity [cps]

0 500 1000 1500 2000

0 10 20 30 40 50 60

t [min]
Figure 3

A. microsomal IRI-G5 formation

B. microsomal IRI-G4' formation

activity [nmol/min/mg protein]

substrate concentration [μM]

activity [nmol/min/mg protein]

substrate concentration [μM]
Figure 5

A. IRI-G5 formation by UGTs

B. IRI-G4' formation by UGTs