Identification of the Human UDP-Glucuronosyltransferases Involved in the Glucuronidation of Combretastatin A-4\footnote{S}

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Received November 27, 2009; accepted April 7, 2010

**ABSTRACT:**

The stilbene compound (Z)-combretastatin A-4 (CA-4) has been described as a potent tubulin polymerization inhibitor. In vivo, CA-4 binds to tubulin and inhibits microtubule depolymerization, which results in morphological changes in proliferating endothelial cells. Combretastatin A-4 prodrug phosphate is a leading vascular disrupting agent and is currently being evaluated in multiple clinical trials as a treatment for solid tumors. The aim of this study was to identify and characterize the UDP-glucuronosyltransferase (UGT) isoforms involved in CA-4 glucuronidation by incubation with human liver microsomes and a panel of nine liver-expressed recombinant UGT Supersomes (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, and 2B17). As we observed, the high rate of formation of CA-4 glucuronide ($V_{\text{max}} = 12.78 \pm 0.29 \text{ nmol/min/mg protein}$) and the low $K_m$ (6.98 ± 0.65 μM) denoted that UGT1A9 was primarily responsible for the in vitro glucuronidation of CA-4. UGT1A6 was also a significant contributor to CA-4 glucuronidation ($V_{\text{max}} = 3.95 \pm 0.13 \text{ nmol/min/mg protein}$ and $S_{\text{opt}} = 44.80 \pm 3.54 \mu M$). Furthermore, we demonstrated that the kinetics of CA-4 glucuronidation with liver microsomes but also with a panel of recombinant UGTs is atypical as it fits two different models: the substrate inhibition and also the sigmoidal kinetic model. Finally, experiments conducted to inhibit the glucuronosyltransferase activity in the human liver microsomes assay showed that phenylbutazone, trifluoperazine, propofol, and 1-naphthol effectively inhibited CA-4 glucuronidation.

**ABBREVIATIONS:** CA-4, (Z)-combretastatin A-4; UGT, UDP-glucuronosyltransferases; CA-4G, (Z)-combretastatin A-4 glucuronide; HPLC, high-performance liquid chromatography; AZT, 3′-azido-3′-deoxythymidine, zidovudine; HLM, human liver microsomes; UDPGA, UDP-glucuronic acid trisodium salt.
tion would help to predict the influence of interindividual variation due to polymorphisms on drug bioavailability, pharmacokinetics, and efficacy. Indeed, because it has been demonstrated (Brown et al., 1995) that CA-4G is characterized by very low pharmacodynamic activity, a different rate of glucuronidation could affect the CA-4 therapeutic effect. Moreover, glucuronidation could also modulate the formation of quinone species whose role in CA-4 pharmacodynamics is at the present time unknown (Aprile et al., 2007). This article describes the full in vitro kinetic characterization of the hepatic UGT isoforms involved in the CA-4 glucuronidation pathway.

Materials and Methods

Chemicals and Reagents. Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich (Milano, Italy). Water (HPLC grade) was obtained from an ELGA PURELAB Ultra system (M-Medical, Milano, Italy). 1-Naphthol, alamethicin, AZT, phenylbutazone, propofol, silybin, trifluoperazine, and UGTs were purchased from Sigma-Aldrich and used without further purification. CA-4 and CA-4G were prepared according to the procedures cited in the literature (Aprile et al., 2009).

Combretastatin A-4 Glucuronidation Assay. Human liver preparations. Human liver microsomes (HLM) (pooled mixed sex, 50 individual donors, protein concentration, 20 mg/ml; total cytochrome P450, 360 pmol/mg protein; rate of formation of estradiol glucuronide (UGT1A1), 1300 pmol/min/mg; trifluoperazine glucuronide (UGT1A4), 730 pmol/min/mg; serotonin glucuronide (UGT1A6), 10,000 pmol/min/mg; propofol glucuronide (UGT1A9), 3300 pmol/min/mg; AZT glucuronide (UGT2B7), 580 pmol/min/mg) were purchased from BD Gentest (Woburn, MA). The incubations were all performed using a horizontal Dubnoff shaking thermostatic bath (DESE-LAB Research, Padua, Italy) and were protected from light.

Recombinant UGTs. Microsomes prepared from baculovirus-infected insect cells that expressed the human UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, and 2B17 (Supersomes; protein concentration 5 mg/ml) were obtained from BD Gentest. The glucuronidation activity of Supersomes was provided by recombinant UGT isoforms (UGT1A4, UGT1A6, UGT2B7, and UGT1A4) and eugenol (UGT2B17).

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HLM and recombinant UGT isoform incubation procedures. Combretastatin A-4 glucuronidation activity was determined both in pooled HLM and in a panel of nine human liver-expressed recombinant UGT isoforms. The conditions for linearity with respect to time were optimized at 0.05 mg/ml protein concentration for HLM and UGT1A9 and at 0.5 mg/ml for the other UGTs. The standard incubation mixture, contained in Eppendorf tubes, was made up of 7.5 to 500 μM CA-4 and 10 mM MgCl₂·6H₂O in a Tris·HCl buffer (50 mM, pH 7.4), which was brought up to a final volume of 150 μl. The final concentration of acetonitrile in the incubation mixture was 1%. An appropriate volume of HLM or UGT suspension, previously activated by alamethicin (50 μg/mg protein) at 4°C for 15 min, was added to give a final protein concentration of 0.5 mg/ml for HLM or UGT1A9 assays and 0.5 mg/ml for other UGTs. After preincubation in a shaking water bath at 37°C, the reaction was initiated by an appropriate volume of UDPGA solution (2 mM final concentration), and the resulting mixture was shaken at 37°C for 9 min in HLM, 10 min in UGT1A1, 1A3, 1A6, 1A9, and 2B17, and 20 min in UGT1A4, 2B4, 2B7, and 2B15 incubations assays. The reaction was quenched by diluting the samples with 37.5 μl of ice-cold formic acid solution (25% v/v). The samples were then centrifuged at 11,000 rpm for 5 min, and the supernatant was directly injected onto the HPLC column for analysis.

Inhibition of HLM, UGT1A9, and UGT1A6 glucuronidation activity. Inhibition of combretastatin A-4 glucuronidation was evaluated using known chemical UGT inhibitors. Incubations were performed using HLM (0.1 mg/ml protein concentration) and 100 μM CA-4 concentration in the same conditions as above, except that the microsome activation was performed using Brij 58 surfactant (0.5 mg/mg protein) in place of alamethicin. Increased concentrations of 1-naphthol (5–500 μM), AZT (50 μM–1 mM), phenylbutazone (5 μM–1 mM), propofol (5 μM–1 mM), silybinin (2.5–100 μM), and trifluoperazine (5–500 μM) were used to inhibit UGT1A6/1A9 (Luukkanen et al., 2005; Fujiwara et al., 2008), UGT2B7 (Picard et al., 2005), UGT1A9 (Kerdpin et al., 2008), UGT1A9 (Picard et al., 2005), UGT1A1 (Sridar et al., 2004), and UGT1A4 (Uchaipichat et al., 2006) isoforms, respectively. Because trifluoperazine is a known selective substrate for UGT1A4, it was used as the putative inhibitor of the same UGT (Fujiwara et al., 2008). Using the same conditions, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. The inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity. Finally, the inhibitory effect of 1-naphthol and trifluoperazine was then evaluated on UGT1A9 and UGT1A6 glucuronidation activity.
line, with respect to analyte stability, selectivity, linearity, accuracy, precision, detection, and quantification limits.

**Stability.** The stability of CA-4G during sample preparation was assessed by treating 150 μl of a CA-4G solution (6.0 μg/ml; 50 mM Tris · HCl, pH 7.4) as reported in the HLM incubation procedure. The supernatant was analyzed in triplicate immediately and after 1 h: no significant difference was observed between peak area values.

**Selectivity.** Method selectivity was assessed with respect to interference arising from biological matrices (i.e., human microsomes, UGT isoforms, and added cofactors) and UGT inhibitors. In the first case, incubations were performed in the absence of CA-4 using the same components as standard incubations; sample preparation and HPLC analysis were performed as reported above. To evaluate the interference of UGT inhibitors and their putative glucuronides, incubations were performed as reported for CA-4 and analyzed by HPLC to evaluate the presence of peaks with the same retention time as CA-4G. In both cases, no interference was observed.

**Linearity.** Linearity was evaluated at nine concentration levels (0.075–10 μg/ml) of CA-4G. These were injected in triplicate, and the peak areas were plotted on an Excel spreadsheet (Microsoft, Redmond, WA). The linear regression equation was generated at nine levels of calibration by least-squares treatment of the data (y = 10057.4x + 3777.5, r² = 0.9999).

**Accuracy.** This was determined at two concentration levels (1.5 and 10 μg/ml) by application of the analytical procedure to recovery studies, where a known concentration of standard CA-4G is spiked in the matrix sample solution. The accuracy studies gave recovery values of 105 and 101% at concentrations of 1.5 and 10 μg/ml, respectively, thus demonstrating that the method was accurate within the desired range.

**Precision.** Precision was evaluated at two concentration levels (1.5 and 10 μg/ml) in terms of instrumental and intraday repeatability as well as interday reproducibility. The instrumental repeatability was assessed by analyzing the same solution six times. The intraday repeatability was investigated using six separate sample solutions that were analyzed in triplicate. The interday reproducibility was checked on four different days, by preparing and analyzing in triplicate four separate sample solutions. The calculated percent relative standard deviation of intraday repeatability and interday reproducibility were always ≤5.0%.

**Limit of detection and quantification.** The limit of detection, determined by injecting progressively low concentration solutions, was 0.025 μg/ml (signal/noise ratio 3). The limit of quantification value was then calculated, resulting in 0.075 μg/ml.

**Results**

We investigated the kinetics of combretastatin A-4 glucuronidation in pooled HLM as well as a panel of nine recombinant UGT Supersomes. CA-4, incubated in the presence of UDP-glucuronic acid, gave the formation of CA-4G, its identity being confirmed by comparison of the retention time and UV spectrum with those of an authentic standard (Aprile et al., 2009). The analyses of incubations were performed as reported above. To evaluate the interference of UGT inhibitors and their putative glucuronides, incubations were performed as reported for CA-4 and analyzed by HPLC to evaluate the presence of peaks with the same retention time as CA-4G. In both cases, no interference was observed.

**Human Liver Microsome Glucuronidation of CA-4.** Human liver microsomes incubated in the presence of CA-4 (7.5–500 μM) generated a significant amount of CA-4G, sufficient to obtain the substrate concentration-glucuronidation velocity curves reported in Fig. 2 and Eadie-Hofstee plots (Supplemental Fig. 1). The corresponding kinetic parameters were determined by fitting the data obtained from microsomal incubations to eqs. 1 to 3 to obtain the correlation coefficient (R²) value. The results of the kinetic analyses showed that CA-4 glucuronidation in HLM fitted the substrate inhibition model (eq. 2), and Vmax, Km, and Kᵢ parameters were thus calculated (Table 1).

**Recombinant UGT Isoform Glucuronidation of CA-4.** Combretastatin A-4 glucuronidation by recombinant UGT Supersomes was investigated using a panel of nine recombinant UGT isoforms (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, and 2B17). CA-4 glucuronidation velocities were evaluated at three different substrate concentrations (50, 100, and 200 μM) and are reported in Fig. 3. UGT1A9 and UGT1A6 exhibited the highest glucuronosyltransferase activity, their velocities (at 100 μM substrate concentration) being 11.8 and 3.3 mmol/min/mg protein, respectively. On the contrary, UGT1A1, A3, 2B7, and 2B17 exhibited low glucuronosyltransferase activity (0.7, 0.7, 0.2, and 0.4 mmol/min/mg protein, respectively). Finally, UGT1A4, 2B4, and 2B15 isoforms showed negligible glucuronosyltransferase activity (<0.03 mmol/min/mg protein). The substrate concentration-glucuronidation velocity curves of recombinant UGTs were also obtained and are reported in Fig. 2, and the Eadie-Hofstee plots are available in Supplemental Fig. 1. The corresponding enzyme kinetic parameters expressed as means ± S.E. are listed in Table 1. As reported for the microsome assay, the best fit and kinetic profile of UGT glucuronidation assays was determined by fitting the data points to eqs. 1 to 3, thus obtaining the R² coefficient value. The first observation was that the kinetic profiles diverge from one enzyme source to another. In the case of UGT1A3, 2B7, 2B15, and 2B17, the data points exhibited a substrate inhibition profile (eq. 2). Moreover, glucuronidation kinetic data for UGT1A1 and UGT1A6 best fitted the sigmoidal kinetic model, their Hill coefficients being n = 0.78 and 1.61, respectively, indicating negative cooperativity (n < 0) or homotropic positive cooperative reaction (n > 0) (eq. 3). In the UGT1A9 glucuronidation assay the data points fitted the substrate inhibition equation well (eq. 2). The observed high rate of formation of CA-4G (Vmax = 12.78 ± 0.29) with the low Km (6.98 ± 0.65) denoted that UGT1A9 was primarily responsible for the in vitro glucuronidation of combretastatin A-4.

**Chemical Inhibition of CA-4 Glucuronidation.** Phenybutazone, propofol, trifluoperazine, and 1-naphthol, when added to the HLM incubation mixture had a relevant and concentration-dependent inhibitory effect. In particular, as shown in Fig. 4, phenybutazone, at a concentration of 1 mM, decreased enzymatic activity by approximately 50%. Likewise, trifluoperazine (500 μM), propofol (1 mM), and 1-naphthol (500 μM) decreased microsomal activity by 12, 22, and 10%, respectively. In addition, we also observed the moderating inhibitory effect of silybin on HLM glucuronidation activity; when this was added to the incubation mixture at a concentration of 100 μM, the microsomal residual activity was found to be approximately 75%. Finally, AZT did not show any inhibitory effect on HLM glucuronidation activity. The inhibitory effect of 1-naphthol and trifluoperazine was also tested on UGT1A9 and UGT1A6 combretastatin glucuronidation. As shown in Supplemental Fig. 2, trifluoperazine inhibited UGT1A9 and UGT1A6 glucuronidation activity to a similar extent as HLM, whereas 1-naphthol only inhibited UGT1A9 similarly to HLM, less affecting UGT1A6 activity. Finally, the inhibitory effects of combretastatin A-4 glucuronide per se on CA-4 glucuronidation by HLM and UGT1A9 were also studied. The addition of CA-4G in CA-4 glucuronidation assays did not affect the enzymatic activity as revealed by the ~100% residual activity (Supplemental Fig. 3).

**Discussion**

Combretastatin A-4 is a new, promising anticancer drug at present undergoing clinical study (Banerjee et al., 2008). The formation of a related glucuronide as the major CA-4 metabolite was demonstrated (Aprile et al., 2009) in rat and human liver fractions as well as in vivo in rat. Several pharmacokinetic studies have shown that the CA-4 phosphate prodrug is rapidly dephosphorylated into the systemic circulation with a very short plasma half-life, with CA-4 and its glucuronide (CA-4G) displaying a longer disposition profile (Dowlati et al., 2002; Rustin et al., 2003). The aim of this study was to identify...
and characterize the UGT isoforms involved in combretastatin glucuronidation by incubation in human liver microsomes and recombinant UGTs Supersomes. First, optimization of the incubation conditions, in terms of linearity of metabolite formation with increased microsomal protein and incubation time was performed with HLM. Second, we examined the activity and kinetics in HLM and then in a panel of nine commercially available recombinant UGT microsomes from baculovirus-infected insect cells. Recombinant UGT1A9 isozyme was the main isozyme catalyzing the glucuronidation of CA-4 (Fig. 3), and the resulting data points fitted the substrate inhibition profile well (eq. 2). UGT1A6 was recognized as making an important contribution to CA-4 glucuronidation. In this instance, the

![Graphs showing enzyme kinetics](image)

**Table 1**

<table>
<thead>
<tr>
<th>Protein Source</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{max}) (nmol/min/mg protein)</th>
<th>(K_i) ((\mu)M)</th>
<th>(S_{50}) ((\mu)M)</th>
<th>Hill Coefficient</th>
<th>Goodness of Fit ((R^2))</th>
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</thead>
<tbody>
<tr>
<td>HLM(^a)</td>
<td>33.26 ± 2.90</td>
<td>41.98 ± 1.66</td>
<td>1126 ± 185.5</td>
<td>228.9 ± 88.5</td>
<td>0.78 ± 0.08</td>
<td>0.99</td>
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<td>UGT1A1(^b)</td>
<td>2.20 ± 0.31</td>
<td>1.53 ± 0.23</td>
<td>295.0 ± 94.4</td>
<td>44.80 ± 3.54</td>
<td>1.61 ± 0.16</td>
<td>0.98</td>
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<tr>
<td>UGT1A4</td>
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<td>N.D.</td>
<td>N.D.</td>
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</tr>
<tr>
<td>UGT1A6(^a)</td>
<td>3.95 ± 0.13</td>
<td>12.78 ± 0.29</td>
<td>2271 ± 490.9</td>
<td>54.80 ± 3.54</td>
<td>1.61 ± 0.16</td>
<td>0.92</td>
</tr>
<tr>
<td>UGT1A9(^a)</td>
<td>6.98 ± 0.65</td>
<td>12.78 ± 0.29</td>
<td>2271 ± 490.9</td>
<td>54.80 ± 3.54</td>
<td>1.61 ± 0.16</td>
<td>0.92</td>
</tr>
<tr>
<td>UGT2B4</td>
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<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>UGT2B7(^a)</td>
<td>4.95 ± 0.64</td>
<td>0.235 ± 0.007</td>
<td>6761 ± 4541</td>
<td>0.90</td>
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<tr>
<td>UGT2B15(^a)</td>
<td>41.74 ± 12.37</td>
<td>0.067 ± 0.009</td>
<td>2182 ± 1900</td>
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<td>UGT2B17(^a)</td>
<td>47.16 ± 12.34</td>
<td>0.70 ± 0.09</td>
<td>1756 ± 1166</td>
<td>0.93</td>
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</table>

N.D., not determined because activity below limit of quantification was observed.

\(^a\) Substrate inhibition model (eq. 2).

\(^b\) Sigmoidal kinetic model (Hill equation) (eq. 3).

![Enzyme kinetics graphs](image)

**Fig. 2.** Enzyme kinetics of CA-4 glucuronidation in HLM and recombinant human UGT isozymes. Data are reported as mean ± S.E.; \(n = 3\).
data points well fitted the sigmoidal kinetic model (Hill profile) (eq. 3), its Hill coefficient being $n > 1$ (Table 1); these features indicated that a positive cooperative reaction had occurred. UGT1A1, which produced moderate glucuronidation activity, also shared the Hill profile, but the Hill coefficient $n < 1$ suggests a negative cooperative reaction. Finally, UGT1A3, 2B7, and 2B17 contributed little to the glucuronidation of CA-4, whereas UGT1A4, 2B4, and 2B15 contributions were negligible (Fig. 3). UGT1A3, 1A9, 2B7, 2B15, and 2B17 exhibited a substrate inhibition profile, thus fitting the data points in eq. 2 well. This atypical kinetic behavior has been described as a two-site model in which one binding site is productive and the other one is inhibitory and operable at a high substrate concentration, resulting in decreased velocity with increased concentration (Hutzler and Tracy, 2002). Alternatively, a more recent kinetic characterization of the UGT1A subfamily suggests that binding of the substrate to the enzyme-UDP complex leads to a nonproductive complex that slows the completion of the catalytic cycle. Furthermore, eq. 2 fits the inhibition data well when it was measured at only one UDPGA concentration (Luukkanen et al., 2005). It is also worth mentioning (Hutzler and Tracy, 2002) that it is important to perform these studies after having eliminated as far as possible any artifactual sources of atypical kinetics such as significant substrate depletion or low substrate solubility. With the express purpose of eliminating these effects, incubations in the presence of HLM and recombinant UGT1A9 needed to be performed using a low protein concentration (0.05 mg/ml) to avoid the total substrate depletion at a low substrate concentration. Moreover, we could not increase the substrate concentration to 500 $\mu$M because the low solubility of CA-4 would have required the use of a high final acetonitrile concentration (>1%) in the incubation buffer. The low protein concentration needed in HLM and the recombinant UGT1A9 assay suggests that they are characterized by high affinity for combretastatin A-4. It is known that the use of a multienzyme system such as HLM may have an impact on the type of kinetics observed (Soars et al., 2003); however, the fact that the substrate inhibition profile was seen throughout the incubations with HLM and five different UGT isoforms indicates that the observed
atypical kinetics is not an artifact. Experiments conducted to inhibit glucuronidation activity identified phenylbutazone, propofol, and 1-naphthol (inhibitors of UGT1A1, IA9, and 1A6/1A9, respectively) as effective inhibitors in HLM combretastatin glucuronidation. The inhibition of CA-4 glucuronidation by trifluoperazine was demonstrated to be due to lack of selectivity of this probe substrate toward UGT1A4. Figure 4 and Supplemental Fig. 2 showed that trifluoperazine (≥100 μM) effectively inhibited UGT1A9 and UGT1A6 combretastatin glucuronidation to a similar extent as HLM. These data are consistent with the negligible UGT1A4 glucuronidation activity revealed in kinetic experiments. Silibinin is known to be a potent inhibitor of UGT1A1, although UGT1A6 and UGT1A9 were also inhibited (Sridar et al., 2004). The moderate capability of silibinin to inhibit CA-4 glucuronidation in HLM (Fig. 4) is consistent with the moderate capability of recombinant UGT1A1 in CA-4 glucuronidation. Finally, the lack of inhibitory potency of AZT on HLM catalyzed CA-4 glucuronidation indicates only a marginal potential for UGT2B7 to contribute to CA-4 glucuronidation. The increasing availability of “probe” substrates and inhibitors for the individual UGTs could enable reliable identification of the UGT(s) responsible for glucuronidation in human liver microsomes. However, when HLM are used as an enzyme source for UGT inhibition studies, the results should be interpreted carefully. Fujiwara et al. (2008) recently emphasized that attention should be paid to the inhibitory effects of UDP on UDP-glucuronosyltransferase activity, which may cause erroneous evaluations in inhibition studies using HLM. In particular, they found that the inhibition of UGT1A1 and UGT1A4 activities in human liver microsomes could be attributed to the inhibitory effect of the UDP produced by UGT1A6-catalyzed 1-naphthol glucuronidation. However, our observation of the prominent inhibition by 1-naphthol in HLM CA-4 glucuronidation could be attributed to the inhibition of UGT1A6 and UGT1A9 because the role of UGT1A1 and UGT1A4 in CA-4 glucuronidation was very moderate as displayed in recombinant isoform glucuronidation assays.

In conclusion, this investigation demonstrated that glucuronidation, the main metabolic pathway of combretastatin A-4, was mainly catalyzed by UGT1A9 and UGT1A6 isoforms, its kinetics being an atypical type.

References

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