UDP-Glucuronic Acid Binds First and the Aglycone Substrate Binds Second to Form a Ternary Complex in UGT1A9-Catalyzed Reactions, in Both the Presence and Absence of Bovine Serum Albumin

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ABSTRACT:
The presence of bovine serum albumin (BSA) largely modulates the enzyme kinetics parameters of the human UDP-glucuronosyltransferase (UGT) 1A9, increasing both the apparent aglycone substrate affinity of the enzyme and its limiting reaction velocity (Drug Metab Dispos 39:2117–2129, 2011). For a better understanding of the BSA effects and an examination of whether its presence changes the catalytic mechanism, we have studied the enzyme kinetics of 4-methylumbelliferone glucuronidation by UGT1A9 in the presence and absence of 0.1% BSA, using bisubstrate enzyme kinetic experiments, in both the forward and reverse directions, as well as product and dead-end inhibition. The combined results strongly suggest that the reaction mechanism of UGT1A9, and presumably other human UGTs as well, involves the formation of a compulsory-order ternary-complex, with UDP-α-D-glucuronic acid (UDPGA) as the first binding substrate. Based on the enzyme kinetic parameters measured for the forward and reverse reactions, the equilibrium constant of the overall reaction was calculated (∆G° = 574) and the relative magnitudes of the reaction rate constants were elucidated. The inclusion of BSA in the bisubstrate kinetic experiments quantitatively changed the apparent enzyme kinetic parameters, presumably by removing internal inhibitors that bind to the binary enzyme-UDPGA (E-UDPGA) complex, as well as to the ternary E-UDPAGA-aglycone complex. Nevertheless, the underlying compulsory-order ternary-complex mechanism with UDPGA binding first is the same in both the absence and presence of BSA. The results offer a novel understanding of UGT enzyme kinetic mechanism and BSA effects.

Introduction

Human UDP-glucuronosyltransferases (UGTs) transfer the glucuronic acid moiety from UDP-α-D-glucuronic acid (UDPGA) to nucleophilic groups on small organic molecules of both endo- and xenobiotic origin (Bock, 2010; Miners et al., 2010). The conjugation with glucuronic acid increases the hydrophilicity of the original aglycone substrate and stimulates excretion from the cell, probably by facilitating molecular recognition by efflux transporters (Jennitz et al., 2010). The amino acid sequences of individual UGT enzymes are highly homologous (Mackenzie et al., 2005), and the enzymes exhibit distinct but partially overlapping substrate and inhibitor specificity (Miners et al., 2010). The UGTs are variably expressed in human tissues, particularly in the liver, intestine, and kidneys (Ohno and Nakajin, 2009; Court et al., 2012). The enzyme at the focus of this study, UGT1A9, is important in the metabolic elimination of several therapeutic drugs, including entacapone (Lautala et al., 2000), propofol (Soars et al., 2004), and mycophenolic acid (Bernard and Guillemette, 2004), and is highly expressed in liver and kidneys (Court et al., 2012).

The presence of purified bovine serum albumin (BSA) in the glucuronidation reaction greatly enhances the in vitro activity of the human UGT1A9, regardless of whether recombinant enzyme or human liver microsomes are used as the enzyme source (Manevski et al., 2011). The inclusion of BSA in both the entacapone and 4-methylumbelliferone (4-MU) glucuronidation assays decreased the $K_m$ values, increased the $V_{max}$ values, and led to some changes in the enzyme kinetic model (Manevski et al., 2011). Such changes in enzyme kinetics open many questions, both practical and theoretical. Two of the more challenging questions are 1) what is the underlying mechanism of the BSA effects and 2) are the results of earlier studies on the UGTs’ reaction mechanism—performed in the absence of BSA—also valid in the presence of this modulator?

The previously suggested BSA-mediated removal of competitive inhibitors, perhaps fatty acids that are released upon cell disruption,
could account for the observed $K_m$ decrease that was found in UGT2B7 (Rowland et al., 2007, 2008). Nevertheless, the removal of competitive inhibitors could not explain the $V_{max}$ increase nor the changes in the enzyme kinetic model that we found in UGT1A9 (Manevski et al., 2011). To account for the latter observations, we proposed that BSA also removes inhibitors that are not directly competing with aglycone substrate binding (Manevski et al., 2011). However, such a hypothesis is poorly defined without knowledge of the reaction mechanism, particularly in enzymes, such as the UGTs, that catalyze a two-substrate, two-product reaction.

The UGTs reaction mechanism has been studied extensively in the last 40 years, yielding variable results (Table 1). Many of these studies found that, regardless of enzyme source and experimental conditions, UGT-catalyzed reactions follow ternary-complex mechanism. The formation of ternary-complex is also supported by the evidence that the glucuronidation reaction resembles $S_{N}2$-type nucleophilic substitution reaction, with the inversion of the glucuronic acid anomeric carbon from $\alpha$- in UDPGA to $\beta$-configuration in the formed glucuronide (Axelrod et al., 1958; Johnson and Fenselau, 1978). Alongside the broad agreement on the formation of ternary complex, however, there is disagreement among the previous studies on whether the two substrates, the aglycone substrate and the UDPGA, bind in a random order or in a compulsory order (Table 1). The observed discrepancies in the order of substrate binding may have arisen from the use of liver microsomal preparations that contain multiple UGT enzymes (Potrepka and Spratt, 1972; Vessey and Zakim, 1972; Sanchez and Tephly, 1975; Rao et al., 1976; Koster and Noordhoek, 1983) or of partially purified UGT enzymes (Matern et al., 1982, 1991; Falany et al., 1987; Yin et al., 1994) that may have been inactivated by detergents during the purification process (Kurkela et al., 2003).

A previous study from this laboratory, which was performed using individual recombinant UGT enzymes of subfamily 1A, concluded that UDPGA is the first binding substrate (Luukkanen et al., 2005). That study was carried out in the absence of BSA and focused on UGT1A9, an enzyme we recently found to be highly affected by the presence of BSA (Manevski et al., 2011). Because of this result, it is particularly interesting to examine whether the presence of BSA affects the reaction mechanism of UGT1A9. To address this question, we have carried out the detailed study that is described below. The results provide comprehensive insights into the UGT reaction mechanism, as well as a theoretical framework for future mechanistic studies with recombinant UGTs.

### Materials and Methods

**Compounds and Reagents.** 4-MU (>99.0%, Chemical Abstract Service (CAS) number 90-33-5), 1-naphthol (>99%, CAS number 90-15-3), UDPGA (ammonium salt, 98–100%, CAS number 43195-60-4), UDP (disodium salt hydrate, ≥96%, CAS number 27821-45-0), 4-methylumbelliferone-$\beta$-D-glucuronide (≥98%, CAS number 6160-80-1), 1-naphthol-$\beta$-D-glucuronide (>99%, CAS number 83833-12-9), sodium phosphate monobasic dihydrate (≥99%, CAS number 13472-35-0), and BSA (≥96%, CAS number 9048-46-8, essentially fatty acid free, ≥0.004%) were purchased from Sigma-Aldrich (St. Louis, MO). Magnesium chloride hexahydrate and perchloric acid were obtained from Merck (Darmstadt, Germany). Formic acid (98–100%) was from Riedel-de Haën (Seelze, Germany). Disodium hydrogen phosphate dihydrate was purchased from Fluka (Buchs, Switzerland). Liquid chromatography-mass spectrometry-grade solvents were used throughout the study.

**Recombinant UGT1A9 and Negative Control Membranes.** Recombinant human UGT1A9 was expressed, as His-tagged protein, in baculovirus-infected Sf9 insect cells (Kurkela et al., 2007). Negative control membranes, called “Bac control,” were prepared from insect cells that were infected with a modified baculovirus that does not encode any functional UGT. Protein concentration in the UGT1A9-enriched and Bac control membranes was determined by the BCA method (Pierce Biotechnology, Rockford, IL).

**Substrate Binding Assays.** The binding of entacapone, 4-MU, and 1-naphthol to 0.1 and 1% BSA, as well as to insect cells membranes, the Bac control membranes, was measured in this study by the rapid equilibrium dialysis system (RED) Thermo Scientific, Rockford, IL. The system consisted of reusable Teflon base plate and single-use device insets comprising two side-by-side vertical cylinder chambers separated by dialysis membrane (molecular weight cutoff value ~8000 Da). The RED device inserts were placed into the base plate, and the sample chamber was filled with 400 μℓ of the test compound solution in 50 mM phosphate buffer (pH 7.4) that also contained the “binding” macromolecules, 0.1 and 1% BSA, or insect cell membranes (0.02–0.2 mg/ml total membrane protein). The buffer chamber was filled with only 600 μℓ of blank buffer and 50 mM phosphate buffer (pH 7.4), without the test compound and the binding macromolecule. The filled up RED device was covered with parafilm and incubated at 100 rpm at 37°C for 6 to 8 h. The test compounds concentration range in the sample chamber was 5 to 750, 5 to 500,

### Table 1

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Aglycone Substrate</th>
<th>Reaction Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig liver microsomes</td>
<td>Bilirubin</td>
<td>Compulsory-order ternary complex (UDPGA first, aglycone second or iso-Theorell-Chance)</td>
<td>Potrepka and Spratt, 1972</td>
</tr>
<tr>
<td>Beef and guinea pig liver microsomes</td>
<td>$p$-Nitrophenol</td>
<td>Rapid equilibrium random-order ternary complex</td>
<td>Vessey and Zakim, 1972</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>Morphine</td>
<td>Compulsory-order ternary complex (UDPGA first, aglycone second)</td>
<td>Sanchez and Tephly, 1975</td>
</tr>
<tr>
<td>Pig kidney microsomes</td>
<td>Estrone</td>
<td>Ternary-complex mechanism, iso-Theorell-Chance (aglycone first, UDPGA second)</td>
<td>Rao et al., 1976</td>
</tr>
<tr>
<td>Purified UGT from rat liver</td>
<td>Chenodeoxycholic acid and testosterone</td>
<td>Ternary-complex mechanism</td>
<td>Matern et al., 1982</td>
</tr>
<tr>
<td>Rat intestinal microsomes</td>
<td>1-Naphthol</td>
<td>Compulsory-order ternary complex (aglycone first, UDPGA second)</td>
<td>Koster and Noordhoek, 1983</td>
</tr>
<tr>
<td>Two purified UGTs from rat liver</td>
<td>Androsterone and testosterone</td>
<td>Rapid equilibrium random-order ternary complex</td>
<td>Falany et al., 1987</td>
</tr>
<tr>
<td>Purified UGT from human liver</td>
<td>Hydoxycholic acid</td>
<td>Ternary-complex mechanism</td>
<td>Matern et al., 1991</td>
</tr>
<tr>
<td>Purified UGT from rat liver microsomes</td>
<td>Substituted phenols</td>
<td>Random-order ternary complex</td>
<td>Yin et al., 1994</td>
</tr>
<tr>
<td>Recombinant human UGTs from 1A family</td>
<td>Entacapone, scopoletin, umbelliferone, 1-naphthol, 4-hydroxyestrone, ethinylestradiol</td>
<td>Compulsory-order ternary complex (UDPGA first, aglycone second)</td>
<td>Luukkanen et al., 2005</td>
</tr>
<tr>
<td>Recombinant human UGT1A6</td>
<td>Scopoletin</td>
<td>Compulsory-order ternary complex (UDPGA first, aglycone second)</td>
<td>Patana et al., 2007</td>
</tr>
</tbody>
</table>
and 0.05 to 5 μM for entacapone, 4-MU, and 1-naphthol, respectively. After incubation, a 50-μl aliquot from each chamber was mixed with 100 μl of ice-cold 4 M perchloric acid/methanol (1:5 mix), placed at −18°C for 30 min, and centrifuged (at room temperature) for 5 min at 16000g, and the test compound concentration in the resulting supernatant was determined by high-performance liquid chromatography (HPLC) analyses. The unbound fraction of the test compound, \( f_u \), was calculated using the following equation:

\[
f_u = \frac{[S]_{\text{buffer chamber}}}{[S]_{\text{sample chamber}}}
\]

where \([S]_{\text{buffer chamber}}\) and \([S]_{\text{sample chamber}}\) are the concentrations of the test compound in the respective chambers at the end of the incubation.

**Glucuronidation Assays.** Stock solutions of 4-MU, 4-MUG, and 1-naphthol were prepared in methanol and diluted with methanol to the desired concentrations immediately before use. Appropriate amounts of these dilutions were transferred into 1.5-ml bench-top centrifuge tubes, and the solvent was evaporated in vacuo at ambient temperature. The solid residues were dissolved in the reaction mixture that was composed of phosphate buffer (50 mM, pH 7.4), MgCl\(_2\) (10 mM), BSA (0 or 0.1%), and the enzyme source (0.02–0.2 mg/ml total membrane protein, either UGT1A9-enriched or Bac control membranes), to a final volume of 100 μl. The concentration of MgCl\(_2\) required for optimal UGT1A9 activity was selected based on literature data (Walsky et al., 2012). Alatameticin was not added because it does not significantly stimulate the glucuronidation activity of recombinant UGT enzymes expressed in S9 insect cells (Kaiivsaar et al., 2008; Zhang et al., 2011; Walsky et al., 2012).

The reaction mixtures, with the dissolved aglycone substrate, were incubated first for 20 min at room temperature and then at 37°C for 5 min. The glucuronidation reaction was initiated by the addition of UDPGA (to a final concentration of 20–5000 μM) and carried out, protected from light, at 37°C for 15 to 60 min, depending on the substrate consumption rate. The reactions in the reverse direction were initiated by the addition of UDP to a final concentration of 5 to 1000 μM. Negative controls, including incubations in the absence of UDPGA (or UDP), without substrate, or with the Bac-control insect cells membranes instead of the UGT1A9-enriched membranes and in the absence of UDPGA (or UDP), without substrate, or with the Bac-control insect cells membranes instead of the UGT1A9-enriched membranes and in the presence of both substrates, were carried out for each set of assays. The glucuronidation reactions in the forward direction were terminated by the addition of 60 μl of ice-cold 4 M perchloric acid/methanol (1:5). To minimize the risk of nonenzymatic glucuronide hydrolysis, the reactions in the reverse direction were terminated by the addition of 100 μl of ice-cold 5% acetic acid in methanol. After reaction termination, the tubes were transferred to −20°C for 60 min and then centrifuged at 16000g for 5 min at room temperature. Aliquots of the resulting supernatants were transferred to dark glass vials and subjected to HPLC analyses.

**Analytical Methods.** The HPLC system consisted of an Agilent 1100 series degasser, binary pump, 100-μl autosampler, thermostated column compartment, multiple wavelengths UV detector, and fluorescence detector (Agilent Technologies, Palo Alto, CA). A Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm; Agilent Technologies) was used for all the separations, at column temperature of 40°C and an eluent flow rate of 1 ml/min. The resulting chromatograms were analyzed with Agilent ChemStation software (revision B.01.01) on Windows XP Professional. Further details of the analytical conditions used for separation and quantification are presented in Table 2.

**Enzyme Kinetic Assays and Data Analysis.** The protein concentrations and incubation times for the kinetic analyses reactions were selected based on preliminary assays so that no more than 10% of the substrate is consumed during the incubation. In the case of bisubstrate enzyme kinetics and inhibition assays, we performed a number of optimization assays to select the suitable concentrations of substrates and inhibitors, as well as protein concentration, incubation time, and optimal parameters of the analytical method for the final assays. Initial velocity measurements in the enzyme kinetic assays in which a concentration of a single substrate was varied were performed in triplicates and are presented as the average ± S.E. Initial velocity measurements for the bisubstrate enzyme kinetics and the inhibition studies were performed in duplicates and are presented as the average value (the variation between two parallel samples in these experiments was not more than 15%, but the differences between the duplicates were much lower than this in most cases). The lines in the Eadie-Hofstee and Lineweaver-Burk plots were plotted with enzyme kinetic parameters obtained from the curve fittings, not by linear regression. Although Eadie-Hofstee plots offer a more reliable presentation of the enzyme kinetics data (Dowd and Riggs, 1965), due to the widespread use of the Lineweaver-Burk “double-reciprocal” plots, we used them to visualize the outcome of product and dead-end inhibition studies. The bisubstrate experiments’ initial rates for the forward reaction catalyzed by the recombinant UGT1A9 were determined by varying the concentration of both substrates simultaneously: the sugar acid donor UDPGA (20–2000 μM) and the aglycone substrate (1–50 and 1–180 μM, with and without substrate inhibition, respectively). The initial rates in the bisubstrate reverse reactions were measured by simultaneously varying the concentration of UDP (5–1000 μM) and 4-MUG (150–10,000 μM). The incubation times were varied from 10 to 60 min. The enzyme kinetic parameters were obtained by fitting the kinetic models to the experimental data, using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., San Diego, CA). The best (most appropriate for each reaction) kinetic model was selected based on visual inspection of the Eadie-Hofstee and Lineweaver-Burk plots, residuals graphs, parameter S.E., and 95% confidence intervals (CIs) estimates, the calculated r² values, and the corrected Akaike’s information criterion. In assays containing BSA, the free substrate or inhibitor concentrations \( f_u \) were corrected according to the measured drug binding to BSA under the specific conditions of each glucuronidation assay (see Substrate Binding Assays above). The initial glucuronidation velocities in the single substrate enzyme kinetics assays were fitted to the following equations. Michaelis-Menten model (eq. 1):

\[
\nu = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]

where \( \nu \) is the initial velocity of the reaction, \([S]\) is the substrate concentration, \(V_{\text{max}}\) is the limiting velocity at saturating concentration of substrate, and \(K_m\) is the Michaelis-Menten constant (concentration of substrate at 0.5 of \( V_{\text{max}} \)).

Substrate inhibition model (eq. 2):

\[
\nu = \frac{V_{\text{max}} [S]}{K_m + [S] \left(1 + \frac{[S]}{K_i} \right)}
\]

where \( K_i \) is the constant describing the substrate inhibition interaction. This model is based on mechanism in which a second molecule of substrate binds.
to preformed enzyme-substrate complex (E-S) and thus acts as uncompetitive inhibitor of the reaction.

For the calculation of enzyme inhibition constants, the obtained data were fitted to the models for competitive (eq. 3), mixed-type (eq. 4), noncompetitive (eq. 5), and uncompetitive inhibition (eq. 6) (Copeland, 2005):

\[
\nu = \frac{V_{\text{max}}[S]}{[S] + K_{a} \left(1 + \frac{[I]}{K_{i}}\right)}
\]

\[
\nu = \frac{V_{\text{max}}}{[S]\left(1 + \frac{[I]}{K_{i}}\right)}
\]

\[
\nu = \frac{V_{\text{max}}[S]}{[S] + K_{a} \left(1 + \frac{[I]}{K_{i}}\right)}
\]

\[
\nu = \frac{V_{\text{max}}[S]}{[S]\left(1 + \frac{[I]}{K_{i}}\right)}
\]

where \(K_{a}, K_{n}, K_{s},\) and \(K_{i}\) are the competitive, mixed-type, noncompetitive, and uncompetitive inhibition constants, respectively. The coefficient \(c\) in eqs. 4 and 6 represents the relative difference in inhibitor’s binding affinity between the free enzyme (competitive modality) and the E-S complex (uncompetitive modality).

The reversible transfer of glucuronic acid from UDPGA to the aglycone substrate is described in this study by the general reaction: \(AX + B \rightleftharpoons AX-B\), in which \(AX, B, BX,\) and \(A\) represent UDPGA, the aglycone substrate, the glucuronic acid conjugate, and UDP, respectively. For the analysis of bisubstrate enzyme kinetic data, the initial velocities were fitted to the following equations:

Compulsory-order ternary-complex model based on a steady-state assumption (Cornish-Bowden, 2012):

\[
\nu = \frac{V_{\text{max}}[AX][B]}{K_{\text{max}}[AX] + K_{\text{mB}}[AX] + [AX][B]}
\]

where \([AX]\) and \([B]\) are the concentrations of UDPGA and aglycone substrate, respectively, \(V_{\text{max}}\) is the limiting velocity at saturating concentration of both AX and B. \(K_{\text{mB}}\) is the equilibrium dissociation constant for the E + AX ⇌ E-AX reaction. \(K_{\text{AX}}\) is the Michaelis constant for AX when B is saturating, and \(K_{\text{mB}}\) is the limiting Michaelis constant for B when AX is saturating. This model assumes that substrate AX binds first and B is the second binding substrate. In the case of the reverse reaction, UDP (A) and 4-MUG (BX) are considered as the first and second substrates, respectively.

The Haldane relationship for the compulsory-order ternary-complex mechanism is given by \(K_{\text{H}} = V_{\text{max}}K_{\text{AX}}K_{AX}/V_{\text{max}}K_{\text{B}}K_{AX}\), where \(V_{\text{max}}\) and \(V_{\text{max}}\) are the limiting velocities in the forward and reverse direction, respectively. Compulsory-order ternary-complex model based on a steady-state assumption with substrate inhibition (Cornish-Bowden, 2012):

\[
\nu = \frac{V_{\text{max}}[AX][B]}{K_{\text{AX}}K_{\text{mB}} + K_{\text{AX}}[AX] + [AX][B]}
\]

where \(K_{\text{AX}}\) is a constant that describes the substrate inhibition interaction. This model assumes that AX and B are first and second enzyme substrates, respectively, and that the inhibition arises from binding of the second substrate, B, to the “wrong” binary complex E-A, instead of E-AX. Compulsory-order ternary-complex model based on a rapid-equilibrium assumption (Copeland, 2000; Alberti, 2011):

\[
\nu = \frac{V_{\text{max}}[AX][B]}{K_{\text{AX}}K_{\text{mB}} + K_{\text{AX}}[AX] + [AX][B]}
\]

where \(K_{\text{AX}}\) is the equilibrium dissociation constant for the E-AX complex (E + AX ⇌ E-AX) and \(K_{\text{mB}}\) is the equilibrium dissociation constant for the E-AX-B complex (E-AX + B ⇌ E-AX-B). This model assumes that AX and B are the first and second binding substrates, respectively. Random-order ternary-complex model based on a rapid-equilibrium assumption (Alberti, 2011):

\[
\nu = \frac{V_{\text{max}}[AX][B]}{K_{\text{AX}}K_{\text{mB}} + K_{\text{AX}}[AX] + K_{\text{AX}}[AX][B] + K_{\text{AX}}[AX][B]}
\]

where \(K_{\text{AX}}\) and \(K_{\text{mB}}\) are the equilibrium dissociation constants for the E-AX and E-AX-B complexes, respectively. \(K_{\text{AX}}\) is the first equilibrium dissociation constant for the E-AX-B complex (E-AX-B ⇌ E-AX + B). \(K_{\text{AX}}\) the second equilibrium dissociation constant for the E-AX-B complex (E-AX-B ⇌ E-B + AX), is not included in this equation but can be calculated from the following relationship:

\(K_{\text{AX}}K_{\text{mB}} = K_{\text{AX}}K_{\text{AX}}\).

Substituted-enzyme (ping-pong bi-bi) model based on a steady-state assumption (Cornish-Bowden, 2012):

\[
\nu = \frac{V_{\text{max}}[AX][B]}{K_{\text{AX}}[B] + K_{\text{AX}}[AX] + [AX][B]}
\]

where \(K_{\text{AX}}\) and \(K_{\text{mB}}\) are the limiting Michaelis constants for AX and B, as in eq. 7. This model also implicitly assumes mechanistically reasonable compulsory-order mechanism, where AX and B are the first and second binding substrates, respectively.

Substituted-enzyme (ping-pong bi-bi) model based on a steady-state assumption with substrate inhibition (Cornish-Bowden, 2012):

\[
\nu = \frac{V_{\text{max}}[AX][B]}{K_{\text{AX}}[B] + K_{\text{AX}}[AX] + [AX][B]}
\]

In this model, substrate inhibition arises from B binding to the free enzyme (E + B ⇌ E-B) and \(K_{\text{mB}}\) represents the dissociation constant for the E-B complex.

**Results**

**Substrates Binding to BSA and Insect Cell Membranes.** The binding of entacapone, 4-MU, and 1-naphthol to BSA, both 0.1 and 1%, and to insect cell membranes, the Bac control membranes that lack any human UGT, was measured in this study by the RED system. The results of the binding assays with this system, in the way we performed them (see Materials and Methods for technical details), were in good agreement with the binding results for entacapone and 4-MU that we previously obtained using ultrafiltration (Manevski et al., 2011) (Fig. 1). For all the tested compounds in this study, the equilibrium was reached within 8 h of incubation in the RED device, under constant shaking. Overnight incubation seemed more susceptible to inaccuracies due to volume shifts, caused by hydrostatic pressure, and increased risk of compounds instability (results not shown). The nonspecific binding to the RED device of the tested compounds was low (≈20%).

Entacapone binds strongly to 0.1% BSA, and, as previously reported (Manevski et al., 2011), the measured \(f_{u}\) for this drug exhibits hyperbolic saturable binding profile (Fig. 1A). The 4-MU binding to 0.1 and 1% BSA was partially concentration-dependent (Fig. 1B), as previously found using the ultrafiltration method (Manevski et al., 2011). The binding of 1-naphthol to 0.1% BSA was rather low and concentration-independent, \(f_{u} = 0.78 \pm 0.01\) (average ± S.E.) (Fig. 1C).

The results of the binding experiments of entacapone, 4-MU, and 1-naphthol to insect cell membranes revealed that in the presence of up to 0.2 mg/ml (total protein), the highest protein concentration used in this work, the binding of all the tested compounds was low. The binding of UDPGA, UDP, and 4-MUG to 0.1% BSA was negligible (data not shown).

**BSA Effects on the Enzyme Kinetics of 1-Naphthol Glucuronidation by UGT1A9.** We have examined 1-naphthol glucuronidation...
The question we tried to answer in this experiment was whether the (low rate) glucuronidation of 1-naphthol by UGT1A9, as well as the usefulness of this compound as inhibitor for UGT1A9, are affected by the presence of BSA in the reaction medium. The addition of 0.1% BSA affected the enzyme kinetics of 1-naphthol glucuronidation by UGT1A9 in a similar manner to its effect on the glucuronidation of entacapone and 4-MU by UGT1A9, namely $K_m$ decrease and $V_{max}$ increase (Manevski et al., 2011). It lowered the $K_m$ of UGT1A9 in the 1-naphthol glucuronidation reaction from $376 \pm 36$ (CI 95%, 302–449) to $177 \pm 14$ nM (CI 95%, 149–206) and increased the reaction’s $V_{max}$ from $0.042 \pm 0.002$ (CI 95%, 0.037–0.048) to $0.068 \pm 0.002$ (CI 95%, 0.064–0.073) nmol · min$^{-1}$ · mg$^{-1}$ (Fig. 2). The results are presented as the average ± S.E., and the calculated 95% CI are given in parenthesis. Although some deviation from the hyperbolic kinetics was observed at low concentrations of 1-naphthol, presumably due to technical difficulties in measuring very low glucuronidation rates, the reaction’s kinetics was, generally, well described by the Michaelis-Menten model ($r^2 \geq 0.98$).

Bisubstrate Kinetics of 4-MU Glucuronidation by UGT1A9. With the aim of understanding the enzyme kinetics mechanism of UGT-catalyzed reactions and how it is affected by the inclusion of BSA, we studied the bisubstrate kinetics of 4-MU glucuronidation by UGT1A9. The results of this analysis, from the perspective of both substrates, are presented as initial velocity versus substrate concentration and as Eadie-Hofstee plots (Fig. 3). The data points were fitted to eqs. 7 to 12 (see Materials and Methods), and the best model was selected based on curve-fitting parameters (see Materials and Methods), experimental evidence, and theoretical considerations (Table 3).

The bisubstrate enzyme kinetics assays, in the absence (as previously done) and presence of BSA, were initially carried out using a lower concentration range of 4-MU, where substrate inhibition is not clearly apparent to the eye (Fig. 3, A and B). The primary Eadie-Hofstee transforms of the data, from the perspective of both substrates, indicated a common intersection point in the second quadrant, left of the y-axis—a pattern characteristic of ternary-complex mechanism. Despite many efforts, however, we did not obtain well defined

by UGT1A9 because, for the purpose of this study, it is a high-affinity but low turnover substrate that can serve as an inhibitor for the aglycone substrate binding (Luukkanen et al., 2005). The question we
intersection points in Fig. 3, A and B. Nevertheless, considering the clearly different intersection point from the one expected in the case of substituted-envelope mechanism (on the x-axis in the first quadrant), we think that our results clearly support the formation of a ternary-complex. The formation of ternary-complex was also supported by the poor fit of the substituted-envelope equation (eq. 11) to the experimental data points. In contrast, the data (Fig. 3, A and B) fitted well to the equations for compulsory-order ternary-complex model based on the steady-state assumption (eq. 7), as well as to the random-order ternary-complex model based on the rapid-equilibrium assumption (eq. 10). The fit to the compulsory-order ternary-complex model, based on the rapid-equilibrium assumption (eq. 9), was not as good, however. The compulsory-order models were also supported by the product and dead-end inhibition studies (see text below and Table 4). The inclusion of BSA did not qualitatively change the bisubstrate kinetics of 4-MU glucuronidation, even if it significantly affected the magnitude of the enzyme kinetic parameters (Table 3). Analysis of the bisubstrate kinetic data with eq. 7 indicates that the inclusion of BSA led to an increase of both the apparent 4-MU affinity (lower $K_m$ value) and the reaction limiting velocity (higher $V_{\text{max}}$). However, the apparent affinity for UDPGA was lower (higher $K_m$ value) in the presence of BSA.

The second set of bisubstrate kinetics reactions was carried out using higher concentrations of 4-MU, in the presence of BSA (Fig. 4). Substrate inhibition became apparent at 4-MU concentrations higher than 40 µM and, interestingly, was also potentiated by higher concentrations of UDPGA. The data fitted well to the steady-state compulsory-order ternary-complex model with substrate inhibition (eq. 8; Table 3), yielding a $K_{\text{ai}}$ value for 4-MU of approximately 50-fold higher than the corresponding $K_m$ value. As in the case of bisubstrate kinetics at lower concentrations of 4-MU (Fig. 3), the lines in the Eadie-Hofstee plots of the bisubstrate kinetics with substrate inhibition showed a common intersection point in the second quadrant (Fig. 4, A and B), the pattern indicative of ternary-complex formation. However, it should be noted that the intersection pattern appears to be different from the perspective of UDPGA at very high concentrations of 4-MU (Fig. 4B, lines marked with dotted line), in the region where substrate inhibition becomes pronounced. This result suggests that potential substrate inhibition by the aglycone substrate should always be taken into account when enzyme kinetics of UDPGA is studied. Otherwise, erroneous conclusions about the enzyme kinetic parameters may be reached.

To understand the overall enzyme kinetic mechanism, we have also investigated the reverse reaction, an approach that was rarely taken in previous UGT studies, particularly because recombinant UGTs became available and ensure that the analysis is conducted on a single enzyme rather than a mixture of different UGTs (Vessev and Zakim, 1972; Rao et al., 1976; Matern et al., 1991). Hence, the bisubstrate enzyme kinetics, in the presence of BSA, was also studied for the reverse reaction, namely the formation of 4-MU and UDPGA from 4-MUG and UDP (Fig. 5). Special attention was paid to the prevention of possible nonenzymatic 4-MUG hydrolysis and to exclude the possibility that other enzymes in the insect cell membrane are catalyzing UDPGA formation from 4-MUG and UDP. The latter was examined using the “Bac-control membranes” that lacked any recombinant UGT, and the assays revealed no significant 4-MU or UDPGA formation upon addition of 4-MUG and UDP. The reverse reaction, in the presence of BSA, was rather fast under optimal conditions, namely in the presence of high concentrations of 4-MUG (Fig. 5; Table 3). The results of the bisubstrate reverse reaction indicated that it follows a ternary-complex mechanism, as can be seen from the common intersection point in the second quadrant of the Eadie-Hofstee plots, just left of the y-axis. It may be noted here that the data points from the reverse reaction are slightly biphasic and, therefore, may indicate a more complex enzyme kinetic model than currently applied (eq. 7). Nevertheless, in the current study, it was important for us to estimate...
### TABLE 3

The bisubstrate enzyme kinetic parameters of 4-MU glucuronidation by UGT1A9

The data were interpreted by compulsory-order, ternary-complex mechanism based on steady-state assumption (eqs. 7 and 8). The values represent a best-fit result ± S.E. The calculated 95% CI are presented in the parenthesis. The reaction velocity is given per milligram of total protein in UGT1A9-enriched insect cell membranes. See Materials and Methods for additional details.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bisubstrate Enzyme Parameters of 4-MU Glucuronidation by UGT1A9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td></td>
<td>$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$</td>
</tr>
<tr>
<td>No BSA</td>
<td>$1.25 \pm 0.04 (1.18-1.33)$</td>
</tr>
<tr>
<td>0.1% BSA</td>
<td>$9.47 \pm 0.13 (9.20-9.75)$</td>
</tr>
<tr>
<td>0.1% BSA with substrate inhibition</td>
<td>$9.44 \pm 0.19 (9.06-9.83)$</td>
</tr>
<tr>
<td>0.1% BSA reverse reaction</td>
<td>$0.872 \pm 0.023 (0.826-0.918)$</td>
</tr>
</tbody>
</table>

### TABLE 4

The inhibition parameters of UGT1A9-catalyzed 4-MU glucuronidation in the presence of 0.1% BSA

The values represent a best-fit result ± S.E. The calculated 95% CI are presented in the parenthesis. The reaction velocity is given per milligram of total protein in UGT1A9-enriched insect cell membranes. See Materials and Methods for additional details.

<table>
<thead>
<tr>
<th>Variable Substrate</th>
<th>Fixed Substrate</th>
<th>Inhibitor</th>
<th>Type of Inhibition</th>
<th>$K_m$ (4-MU)</th>
<th>$V_{\text{max}}$</th>
<th>$K_i$ (4-MU)</th>
<th>$\alpha$</th>
<th>Goodness-of-fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-MU</td>
<td>UDPGA (2000)</td>
<td>UDP</td>
<td>Mixed</td>
<td>$2.97 \pm 0.14 (2.68-3.26)$</td>
<td>$9.56 \pm 0.11 (9.33-9.79)$</td>
<td>$121 \pm 12 (98.1-145)$</td>
<td>$2.92 \pm 0.43 (2.07-3.77)$</td>
<td>0.99</td>
</tr>
<tr>
<td>4-MU</td>
<td>UDPGA (2000)</td>
<td>1-Naphthol</td>
<td>Mixed</td>
<td>$3.20 \pm 0.25 (2.71-3.69)$</td>
<td>$7.73 \pm 0.16 (7.43-8.04)$</td>
<td>$0.0198 \pm 0.0027 (0.0153-0.0243)$</td>
<td>$7.38 \pm 1.75 (3.91-10.9)$</td>
<td>0.98</td>
</tr>
<tr>
<td>UDPGA</td>
<td>4-MU (30)</td>
<td>UDP</td>
<td>Competitive</td>
<td>$117 \pm 5 (107-126)$</td>
<td>$8.78 \pm 0.08 (8.62-8.93)$</td>
<td>$19.1 \pm 0.8 (17.5-20.6)$</td>
<td>$0.0015 \pm 0.0658 (0.0628-0.0689)$</td>
<td>0.99</td>
</tr>
<tr>
<td>UDPGA</td>
<td>4-MU (30)</td>
<td>1-Naphthol</td>
<td>Uncompetitive</td>
<td>$137 \pm 5 (127-147)$</td>
<td>$9.10 \pm 0.09 (8.93-9.28)$</td>
<td>$0.0658 \pm 0.0015 (0.0628-0.0689)$</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>
the enzyme kinetic parameters of the reverse reaction in the context of the overall enzyme kinetic mechanism. From this point of view, the equation of compulsory-order ternary-complex reaction mechanism serves as a good approximation, even if we cannot exclude at this stage a more complex behavior. Future studies may address this issue in more detail. The \( K_m \) value for 4-MUG was approximately 500-fold higher than the \( K_m \) value for 4-MU, whereas the \( K_v \) value for UDP was approximately one order of magnitude lower than the corresponding value for UDPGA. Based on the Haldane relationship for the compulsory-order ternary-complex mechanism (see Materials and Methods), the thermodynamic equilibrium constant of the reaction in the presence of BSA is \( K_{eq} = 574 \).

One can expect unique relationships between the enzyme kinetic parameters and the individual rate constants in the compulsory-order ternary-complex mechanism (Cornish-Bowden, 2012). Unfortunately, due to lack of purified and fully active UGT1A9 enzyme, we could only determine the relative ratio of the rate constants, not the absolute values. For comparison, the results were normalized by arbitrarily setting the value of \( V_{max}^r \), the limiting reaction velocity in the forward direction, to 1 and then using the expression \( V_{max}^r = k_{cat}^r [E] \), where [E] is the molar concentration of the enzyme (Table 5). It is worth noting here that although the first-order and second-order rate constants cannot be directly compared, a pseudo first-order rate constant such as \( k_1[A]X \) can be compared with other first-order constants. These results indicate that the catalytic rate constant in the forward direction, \( k_{cat}^r \), is approximately 11-fold higher than the corresponding catalytic rate constant in the reverse reaction \( k_{cat}^i \).

Inhibition Studies of UGT1A9-Catalyzed 4-MU Glucuronidation in the Presence of 0.1% BSA. The bisubstrate enzyme kinetic experiments confirmed the existence of ternary-complex. To distinguish between compulsory-order and random-order mechanism, we performed product inhibition studies with UDP and dead-end inhibition studies with 1-naphthol. As noted above, 1-naphthol is a high-affinity but low turnover substrate for UGT1A9 (\( K_m = 177 \pm 14 \text{ nM} \); \( V_{max} = 0.068 \pm 0.002 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \)) and, under specific experimental conditions, can be regarded as a 4-MU-competing dead-end inhibitor of UGT1A9.

The results revealed that UDP is a mixed-type and competitive inhibitor of 4-MU glucuronidation with respect to 4-MU and UDPGA, respectively (Figs. 6A and 7A; Table 4). On the other hand, 1-naphthol was found to be a mixed-type inhibitor with respect to 4-MU (Fig. 6B; Table 4), although with the competitive component of the inhibition clearly prevailed (\( \alpha = 7.38 \pm 1.75; \text{average } \pm \text{ S.E.} \) (Table 4). More importantly, however, 1-naphthol was an uncompetitive inhibitor with respect to UDPGA (Fig. 7B; Table 4). In addition, it may be added that the determined \( \alpha \) value of 1-naphthol’s \( K_m (177 \pm 14 \text{ nM}) \) and mixed-type \( K_i \) with respect to 4-MU (19.8 \pm 2.7 \text{ nM}) are not in good agreement with each other. The observed discrepancy may be due, at least partially, to the different physical meaning of the \( K_m \) and \( K_i \) values. Although the \( K_i \) is a true equilibrium-dissociation constant of the enzyme-1-naphthol complex (or in the case of compulsory-order substrate binding, the enzyme-UDPGA-1-naphthol complex), the \( K_m \) is a pseudo affinity constant that may or may not equal the true substrate dissociation constant.

**Discussion**

We recently found that the “albumin effect” in human UGT1A9 is different in UGT2B7, because it both decreases the \( K_m \) and increases the \( V_{max} \) in the 4-MU and UGT1A9-catalyzed entacapone glucuronidation reactions (Manevski et al., 2011). This finding prompted us to
reexamine the reaction mechanism of UGT1A9 and whether it is affected by the presence of BSA. We selected 4-MU as an aglycone substrate for the detailed enzyme kinetic analysis because it is a high-affinity, high-turnover substrate and exhibits substrate inhibition at high concentrations, a feature typical for many UGT-catalyzed reactions. In addition, 4-MU and 4-MUG are commercially available and, importantly, are highly fluorescent, making them suitable for sensitive detection.

For accurate analysis of the BSA effect, it is essential to determine the free drug concentration in the presence of the used BSA concentration. For such measurements, we have now used the RED method (Waters et al., 2008) and compared the results with our previous data that were obtained using ultrafiltration (Manevski et al., 2011). The two methods provide very similar results for both entacapone and 4-MU binding to BSA, when taking into account the nonspecific drug binding to the device (Fig. 1). The inclusion of MgCl₂, UDPGA, or UDP did not significantly alter the substrates binding to BSA. In contrast, the addition of membrane samples (0.02–0.2 mg/ml) significantly modulated the entacapone binding to 0.1% BSA and should be taken into account (Manevski et al., 2011).

To examine whether BSA affects the reaction mechanism of UGT1A9, we have used bisubstrate kinetic analysis of 4-MU glucuronidation by UGT1A9. The results, both in the forward and the reverse directions, strongly suggested that the 4-MU glucuronidation reaction proceeds through the formation of ternary-complex, regardless of whether BSA was added to the assay (Figs. 3–5; Table 3). The bisubstrate kinetic data were best described by a steady-state compulsory-order ternary-complex model, even though evidence from complementary inhibition experiments may be needed to fully prove this (see below). BSA presence affected the forward reaction by lowering the $K_m$ value for 4-MU and increasing the $V_{max}$ value. In addition, and somewhat surprisingly, it increased the $K_m$ value of UGT1A9 for UDPGA (Fig. 3; Table 3). However, the inclusion of BSA did not

### Table 5

The relative individual rate constants for compulsory-order, ternary-complex mechanism of UGT1A9-catalyzed 4-MU glucuronidation

<table>
<thead>
<tr>
<th>Rate Constant Equation</th>
<th>Relative Value, Normalized to $k_{cat}$ = 1</th>
<th>Order of Rate Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}}{K_{max}}$</td>
<td>Second-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}K_{max}}{V_{max}}$</td>
<td>First-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}(k_{cat} + k_{1})}{k_{1} + K_{F}}$</td>
<td>Second-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}V_{max}K_{F}}{V_{max}V_{max}K_{F} - V_{max}V_{max}K_{max}}$</td>
<td>First-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}V_{max}K_{F}}{V_{max}V_{max}K_{max}}$</td>
<td>Second-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}V_{max}K_{F}}{V_{max}V_{max}K_{max}}$</td>
<td>First-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}V_{max}K_{F}}{V_{max}V_{max}K_{max}}$</td>
<td>Second-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}V_{max}K_{F}}{V_{max}V_{max}K_{max}}$</td>
<td>First-order</td>
</tr>
<tr>
<td>$k_{cat}[E]$</td>
<td>$\frac{v_{max}V_{max}K_{F}}{V_{max}V_{max}K_{max}}$</td>
<td>Second-order</td>
</tr>
</tbody>
</table>

![A] 4-MU kinetics in the presence of 0.1% BSA, inhibition by UDP

![B] 4-MU kinetics in the presence of 0.1% BSA, inhibition by 1-naphthol

![A1] Inhibition of the UGT1A9-catalyzed 4-MU glucuronidation reaction by UDP (A) and 1-naphthol (B). The reactions were carried out in the presence of 0.1% BSA, and the points represent the average of two samples (variation between two replicates was less than 15%). The glucuronidation rates are presented as measured initial rates in nmol min⁻¹ mg⁻¹ of UGT1A9-enriched insect cell membranes. The derived kinetic and inhibition constants are presented in Table 4. The Lineweaver-Burk transformations of the data are presented in A1 and B1.
qualitatively affect the bisubstrate kinetics model (Fig. 3, see Eadie-Hofstee insets).

The analysis of bisubstrate kinetics results in the forward and reverse directions, when both were assayed in the presence of BSA, revealed that UGT1A9 has approximately 500-fold higher affinity for 4-MU than for 4-MUG (Table 3). It is interesting to note that the apparent affinity of the enzyme for UDP, as measured in the reverse reaction, is an order of magnitude higher than for UDPGA in the forward reaction (Table 3). Taken together, the results suggest that the reverse reaction is mainly limited by the poor affinity of the enzyme for 4-MUG. Another factor that contributes to making the reaction proceed almost exclusively in the forward direction in the human cell is that the $k_{-2}$ (first-order rate constant for $E$-$AX-B \rightarrow E$-$AX + B$ reaction) is much smaller than $k_1$ (first-order rate constant for $E$-$AX-B \rightarrow E$-$A + BX$ reaction) (Fig. 8; Table 5). A comparison of the relative individual rate constants also shows that the first-order rate constants in the forward direction, such as $k_3$ and $k_4$, are within the same order of magnitude as $k_{-1}$, an observation that supports the steady-state assumption (Fig. 8; Table 5). Based on the measured enzyme kinetic parameters and the Haldane relationship, the equilibrium constant of the overall reaction is large, $K_{eq} = 574$. Nevertheless, as we clearly show in this study (Fig. 5), the reverse reaction can also be performed and analyzed under, more or less, conditions of standard in vitro UGTs assays.

The presence of substrate inhibition at high concentrations of 4-MU (Fig. 4), which appears to be stimulated by the presence of high UDPGA concentrations, provides evidence for a steady-state compulsory ternary-complex reaction mechanism (see also Luukkanen et al., 2005). In this mechanism, either the second substrate or the first product of the reaction may bind to a wrong binary complex. The results of the bisubstrate kinetic analyses support the assumption that, in the case of the studied reaction, UDPGA and 4-MU are the first and
second binding substrates, respectively, and that substrate inhibition probably occurs as a result of 4-MU binding to a wrong binary complex, E-UDP, rather than to the “correct” binary complex, E-UDPGA (Fig. 8). The measured relatively high affinity of UDP to the free enzyme may explain why substrate inhibition is commonly observed in UGT-catalyzed reactions, especially if the initial rate conditions are not strictly followed and the UDP concentration increases due to its accumulation during the forward reaction (Table 3). This reaction should raise awareness about potential problems in substrate depletion experiments with the UGTs. In such experiments, contrary to initial-rate assays, the accumulation of UDP is likely to slow down the aglycone substrate depletion.

Substrate inhibition can also occur in the random-order ternary-complex mechanism, where 4-MU may also bind to E-UDP complex. However, if the rapid-equilibrium assumption is valid, the E-UDP complex concentration is zero in the absence of accumulated or added products. Because 4-MU cannot bind to enzyme species that are not present, the rapid-equilibrium random-order ternary-complex mechanism can be excluded based on this observation. However, the non-rapid-equilibrium random-order ternary-complex mechanism may not be ruled out based on bisubstrate kinetics only. However, the rapid-equilibrium compulsory-order ternary-complex may be excluded based on herein measured and reported rate constant magnitudes (Table 5) (Luukkanen et al., 2005, substrate inhibition, and poor fit to experimental data.

UDP was found to be a competitive inhibitor with respect to UDPGA, and mixed-type inhibitor was competitive with respect to 4-MU (Fig. 6, A and A1; Table 4). Such inhibition patterns agree well with the previously published data (Luukkanen et al., 2005; Fujiwara et al., 2008) and are possible for both random-order and compulsory-order ternary-complex mechanisms. However, if the reaction follows the compulsory-order ternary-complex mechanism, as indicated by the bisubstrate kinetic analyses and substrate inhibition, the UDP inhibition results support the suggestion that UDPGA is the first binding substrate in a compulsory-order mechanism.

In line with previous finding in the absence of BSA (Luukkanen et al., 2005), 1-naphthol is a predominantly competitive inhibitor of UGT1A9 with respect to 4-MU (α = 7.38 ± 1.75) but, importantly, uncompetitive with respect to UDPGA (Fig. 6, B and B1; Table 4). This result indicates that 1-naphthol, an inhibitor that probably competes with 4-MU for the aglycone substrate-binding site, does not compete for the same enzyme species as UDPGA. The uncompetitive inhibition pattern arises from 1-naphthol binding to the preformed E-UDPGA complex, rather than to the free enzyme. This uncompetitive inhibition provides strong evidence that UGT substrates are binding in a compulsory fashion in which the initial binding of UDPGA increases the affinity for the aglycone substrate. Such an affinity increase could be a result of a conformational change in the enzyme upon UDPGA binding, the involvement of the bound UDPGA molecule itself in the binding of the aglycone substrate (see Fig. 9 of Itaiko et al., 2010), or both. We can also expect that the reverse reaction should be structurally analogous to the forward reaction and that the second product of the forward reaction (UDP) should be the structural analog of the first substrate (UDPGA).

Based on the available evidence, UGT1A9 follows a steady-state compulsory-order ternary-complex mechanism, regardless of whether BSA is present (Fig. 8). One may then ask how BSA interferes with this reaction mechanism to affect the apparent substrate affinities and the reaction Vmax. Taking into account that the presence of BSA increases the apparent affinity for 4-MU and decreases the apparent affinity for UDPGA, it may be proposed that BSA removes internal inhibitors that are competitive and/or noncompetitive with respect to the aglycone substrate, but internal inhibitors are uncompetitive with respect to UDPGA. The uncompetitiveness of the BSA-removed inhibitors with respect to UDPGA would explain why the affinity for this cosubstrate apparently decreases in the presence of BSA. Such inhibitors, tentatively marked as I1 and I2 in the reaction scheme (Fig. 8), would not (or poorly) bind to the free enzyme, but they would bind with higher affinity to the binary E-UDPGA complex or the ternary E-UDPGA-4-MU complex.

If the UGT1A9 inhibitor(s) source is the membrane in which the enzyme is located, it may be suggested that the differences in lipid composition between the insect cell membranes (see Marheineke et al., 1998 for the lipid composition of Sf9 membranes) to the human liver membranes could lead to differences in the BSA effects between the recombinant UGT and the native enzyme. At this time, we cannot discard this possibility, particularly regarding the magnitude of the BSA effect when different substrates are used. Nevertheless, recent studies on the BSA effects demonstrated only minor influences of the membrane source on the BSA effects in UGT1A9 (Manevski et al., 2011; Shiraga et al., 2012) or UGTs 1A1, 1A4, 1A6, and 2B7 (Walsky et al., 2012). Hence, the currently available results do not indicate significant differences between Sf9 membranes and human liver microsomes as sources of the inhibitory fatty acids that are removed by BSA, when present during the glucuronidation reaction. Although the exact nature and number of these inhibitors is currently unknown, the improved understanding of the UGT reaction mechanism and the BSA effects may help to rationalize and predict the BSA effect in future.

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Authorship Contributions
Participated in research design: Manevski and Finel.
Conducted experiments: Manevski.
Performed data analysis: Manevski.
Wrote or contributed to the writing of the manuscript: Manevski, Finel, and Yli-Kauhaluoma.

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