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Title page

The Role of Human UDP-Glucuronyltransferases in the Biotransformation of the Triazolo- and Imidazooacridinone Antitumor Agents C-1305 and C-1311: Highly Selective Substrates for UGT1A10.

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Running title:

Glucuronidation of antitumor acridinones

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Nonstandard abbreviations:

FMO, Flavin-containing Monooxygenase

GI, Gastrointestinal

HIM, Human Intestinal Microsomes

HLM, Human Liver Microsomes

RLM, Rat Liver Microsomes

UGT, UDP-glucuronosyltransferase

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ABSTRACT

5-Diethylaminoethylamino-8-hydroxyimidazoacridinone, C-1311 (NSC-645809), is an antitumor agent shown to be effective against breast cancer in phase II clinical trials. A similar compound, 5-dimethylaminopropylamino-8-hydroxytriazoloacridinone, C-1305, shows high activity against experimental tumors and is expected to have even more beneficial pharmacological properties than C-1311. Previously published studies showed that these compounds are not substrates for cytochromes P450; however, they do contain functional groups that are common targets for glucuronidation. Therefore, the aim of this work was to identify the human UDP-glucuronosyltransferases (UGTs) able to glucuronidate these two compounds. High pressure liquid chromatography (HPLC) analysis was used to examine the activities of human recombinant UGT1A and UGT2B isoforms and microsomes from human liver (HLM), whole human intestinal mucosa (HIM) and seven isolated segments of human gastrointestinal (GI) tract. Recombinant extrahepatic UGT1A10 glucuronidated 8-hydroxyl groups with the highest catalytic efficiency as compared to other recombinant UGTs, V_{max}/K_m = 27.2 and 8.8 µl/min/mg protein, for C-1305 and C-1311, respectively. In human hepatic and intestinal microsomes (HLM and HIM), high variability in UGT activities was observed among donors and for different regions of intestinal tract. However, both compounds underwent UGT-mediated metabolism to 8-O-glucuronides by microsomes from both sources with comparable efficiency, V_{max}/K_m values were from 4.0 to 5.5 µl/min/mg protein. In summary, these studies suggest that imidazo- and triazoloacridinone drugs are glucuronidated in human liver and intestine *in vivo* and may form the basis for future translational studies of the potential role of UGTs in resistance to these drugs.

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INTRODUCTION

Among several groups of diaminoalkyloacridines with heterocyclic aromatic rings condensed with an acridine core, triazolo- and imidazocridinones exhibited the most potent antineoplastic properties toward a wide spectrum of transplantable tumors (Konopa, 2001) (Fig. 1). Triazoloacridinones exhibited significant and clearly differentiated cytotoxic activity in vitro toward 64 human tumor cell lines in the NCI screening system (Bethesda, MD, USA) and also displayed high antitumor activity against several experimental tumors in mice, particularly leukemias and colon carcinomas (Chołody *et al*., 1990a; Kuśnierczyk *et al.*, 1994). One highly active and promising derivative of this group, C-1305 (5-dimethylaminopropylamino-8-hydroxytriazoloacridinone), has been selected for extended preclinical trials. The best known imidazoacridinone analogue, C-1311 (5-diethylaminoethylamino-8-hydroxyimidazoacridinone, NSC-645; Chołody *et al.,* 1990b) has shown potent activity against experimental models of murine and human colorectal cancer in vitro and in animals (Burger *et al.*, 1996). It was evaluated in phase I clinical trials in patients with advanced solid tumors. (Thomas *et al.*, 2008, Isambert *et al.*, 2010) and was effective in a phase II clinical trial in women with metastatic breast cancer (Capizzi *et al*., 2008). The most recent studies have shown that C-1311 in combination with paclitaxel was effective against human bladder cancer in the *in vivo* hollow fiber assay (Smith *et al.,* 2011)

The biological and biochemical mechanisms of C-1305 and C-1311 action are under investigation (Mazerska *et al.*, 2001; Mazerska *et al.*, 2003; Augustin *et al.*, 2006; Skwarska *et al.*, 2007). It has been shown that both compounds intercalate to DNA, however, physicochemical DNA binding is not crucial for the observed antitumor activity of these compounds (Dzięgielewski *et al.*, 2002; Koba and Konopa, 2007). It is postulated that the anticancer properties of these compounds are related to their inhibition of topoisomerase II activity (Lemke *et al.*, 2004; Składanowski *et al.*, 1996) and interstrand covalent DNA cross-linking in tumor cells by metabolically activated compounds (Dzięgielewski and Konopa, 1996; Koba and Konopa, 2007).

Taking into account the suggested role of metabolism in the ability of these compounds to covalently bind DNA (Mazerska *et al.*, 2001; Koba and Konopa, 2007) this work attempts to elucidate the metabolic pathways of these agents. It has been shown previously that, although cytochrome P450s are not involved in C-1305 or C-1311 activation, both compounds are selective irreversible inhibitors of CYP1A2 and CYP3A4 but not CYP2 isoforms. (Potęga *et al.*, 2011, Fedejko-Kap *et al*.,

2011). Each compound was also found to be metabolized by rat liver microsomes (RLM) and HLM to N^{ω}-oxide derivatives in the aminoalkyl side chain. Identical metabolites were found to be products in reactions with the human recombinant flavin-containing monooxygenases, FMO1 and FMO3. Therefore, N^{ω}-oxide derivatives of C-1305 and C-1311 are believed to be the FMO-mediated products of RLM and HLM (Potega *et al.*, 2011, Fedejko-Kap *et al*., 2011).

Conjugative metabolism has also been investigated and UGTs, have been identified as being involved in the biotransformation of C-1311. These studies revealed that C-1311 undergoes O-glucuronidation by RLM and recombinant UGT1A1 but not UGT2B7 (Potega *et al.*, 2011). Glucuronidation of C-1311 has been shown to also occur in mice, with high levels of C-1311 glucuronide found in both plasma and liver (Calabrese *et al.*, 1999). Therefore, the present work evaluates the role of human UGTs in the metabolism of C-1311 and C-1305 using a set of recombinant UGTs, HLMs, and HIMs. UGTs exhibit tissue specific expression patterns. As such, the UGT enzymes selected for screening will shed light on the glucuronidation in extrahepatic tissues.

This work is expected to result in the generation of information concerning how minor structural differences between triazolo- and imidazoacridinone moieties of these compounds will affect their glucuronidation. Moreover, the results obtained will also provide information on the pharmacokinetics of C-1305 and C-1311 in human liver and intestine. The identification of specific human UGT isoforms will allow us to focus on their role in drug resistance and/or regulation of toxicity in cancer cells.

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MATERIALS AND METHODS

Chemicals

C-1305 and C-1311 and their 8-methoxy derivatives were synthesized in the Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology, as described earlier (Cholody *et al*., 1990a, Cholody *et al*., 1990b, Cholody *et al*., 1992). All chemicals used were of at least reagent grade. Ethyl alcohol (95%) was purchased from AAPER (Shelbyville, KY). Ammonium formate and formic acid were from Fisher Scientific Co. (Thermo Fisher Scientific, Waltham, MA). UDP-GlcUA Na_3 salt, β-glucuronidase (GUS) and all other chemicals and reagents, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Enzymes

Recombinant human UGT1A1, 1A3, 1A4, and 1A6–1A10 were produced in baculovirus-infected insect cells as described previously (Kurkela *et al*., 2007). The expression level of individual recombinant UGTs was estimated by Western blot analysis using monoclonal antibodies (Tetra-His antibodies; QIAGEN GmbH, Hilden, Germany) against the His-tag that all of them carry (Kurkela *et al*., 2007). For activity comparison between individual UGTs, the enzyme levels were normalized as described previously (Kurkela *et al*., 2007). Human UGT2B4, 2B7, 2B15 and 2B17 (5 mg of protein/ml), expressed in baculovirus-insect cells (supersomes^{TM}), were purchased from BD Biosciences (Woburn, MA). Each enzyme tested in this study is known to be active towards substrates specific for that isoform. Human liver microsomes (HLM) and human intestinal microsomes (HIM) were obtained as described previously (Antonio *et al*., 2003; Sabolovic *et al*., 2004). Human intestinal and liver tissues were obtained from organ donors by transplant surgeons at University Hospital, Little Rock, AR, according to a protocol approved by the Human Research Advisory Committee of the University of Arkansas for Medical Science and were prepared as described previously (Antonio *et al*., 2003; Sabolovic *et al*., 2004).

Screening of Human Liver and Intestinal Microsomes and Recombinant UGT Isoforms.

HLM and HIM (20 µg protein), each from a single donor, or recombinant UGT isoform protein (5 µg protein) were assayed for activity toward C-1305 and C-1311 as follows. The proteins were incubated in a buffer containing 100 µM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM saccharolactone and 2% DMSO supplemented with either 100 or 200 µM substrate in a total volume of 30 µl. (2% DMSO

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was added to permeabilize the membrane; Zielinska *et al*. 2008). Substrates were added in water. Reactions were started by the addition of UDP-GlcUA (3 mM) and incubated for 60 min at 37°C. The reactions were stopped by the addition of 30 µl of ethanol, followed by centrifugation at 12,000 x g for 8 min to pellet the denatured protein. The supernatant fractions were used for HPLC analysis. Control reactions omitting substrate were run with each assay. All incubations were performed in triplicate. Hydrolysis with β-glucuronidase was used to identify the glucuronide peaks. For this purpose, after incubation as described above, 1000 U of β-glucuronidase (type VII-A from *Escherichia coli*) was added and samples were incubated for additional 60 min and processed as above.

High-Performance Liquid Chromatography-UV/Vis Analysis.

HPLC analyses of the supernatants were performed using an HP1050 HPLC system and the Agilent ChemStation software package. Samples were separated using a reversed-phase 5 µm Suplex pKb-100 analytical column (0,46 cm x 25 cm, C18) (Supelco, USA) warmed to 25°C. The analyses were carried out at a flow rate of 1ml/min with the following mobile phase system: a linear gradient from 15 to 80% methanol in ammonium formate buffer (0.05 M, pH 3.4) for 25 min., followed by a linear gradient from 80 to 100% methanol in ammonium formate for 3 min. The column was then re-equilibrated at initial conditions for 10 min between runs. The elution of each metabolite was monitored at 420 nm. Primary standards for the glucuronidated metabolites of C-1305 and C-1311 are not available, however, it was demonstrated that the GlcUA moiety does not alter the absorption maximum for C-1305 at 420 nm (Fig. 6), as has been shown previously for C-1311 (Potega et al., 2011). Therefore, product concentrations were calculated using the response for substrate. The calculated limits of detection (LOD) were equal to 0.504 and 0.357 µM for C-1305 and C-1311, respectively. The reproducibilities (%RSD – Relative Standard deviations) were 1.2% for C-1305 and 1.0% for C-1311.

Liquid Chromatography-Tandem Mass Spectrometry Analysis.

HPLC-MS/MS analyses of the products were carried out by electrospray ionization with positive ion detection and an Agilent 1100 LS-MSD mass spectrometer (Agilent Technologies, USA). Samples were separated as described above for HPLC analyses.

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Steady State Enzyme Kinetics Assays.

Kinetic parameters were determined by incubation of recombinant UGT membrane protein (5 µg) or microsomes (20 µg) in the presence of varying concentrations of the substrate (5 to 1000 µM) at a fixed concentration of UDP-GlcUA (3 mM) for 60 min. All other conditions were identical to those of the screening experiments. Kinetic data for the glucuronidation of C-1305 and C-1311 by UGTs were estimated by plotting the measured initial reaction velocity values as a function of substrate concentration and fitting these to the Michaelis-Menten equation (1), Hill equation (2) or uncompetitive substrate inhibition model (3), using Prism4 software (Graph Pad Software, San Diego, CA.):

$$V = V_{max} \cdot S / (K_m + S) \quad (1)$$

$$V = V_{max} \cdot S^n / (S_{50}{}^n + S^n) \quad (2)$$

$$V = V_{max} / (1 + K_m / S + S/K_{si}) \quad (3)$$

where K_m is a Michaelis-Menten constant, V_{max} is the maximum velocity, S_{50} is the substrate concentration at 50% V_{max} (analogous to K_m in Michaelis-Menten kinetics), n is the Hill coefficient, which can be considered to be a measure of autoactivation and reflects the extent of cooperativity among multiple binding sites, and K_{si} is the inhibition constant.

RESULTS

Glucuronidation of C-1305 and C-1311 by human recombinant UGTs.

Recombinant human UGT1A isoforms expressed in Sf9 cells as His-tag proteins and four human recombinant UGT2B isoforms (Supersomes™) were screened for their ability to glucuronidate C-1305 and C-1311. For these preliminary screenings, two concentrations (100 and 200 µM) of each drug were used (Fig. 2) and the formation of a single metabolite of each was shown (Fig. 3). The purpose of these experiments was to identify which human isoforms are involved in the conjugation of these two drugs, as well as to determine what range of substrate concentrations should be used for kinetic analysis. Screening data indicated that both compounds were glucuronidated by UGT1A1 and 1A3 at very low levels (< 0.1 nmol/mg protein/min), by UGT1A7-1A9 isoforms to a greater extent (0.2 - 0.3 nmol/mg protein/min) and the glucuronidation rate with UGT1A10 was the highest for both compounds (> 0.5 nmol/mg protein/min). UGT1A7 was moderately active toward C-1305 but showed only a minor

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activity toward C-1311. UGT1A10 glucuronidated C-1305 at a rate nearly three times higher than with C-1311. No UGT2B isoforms had measurable activity with either of the substrates tested.

Glucuronidation of C-1305 and C-1311 by HLM and HIM.

Since both drugs were found to be glucuronidated by isoforms known to be expressed in both hepatic (1A1 and 1A9) and extrahepatic tissues (1A7 and 1A10) (Harbourt *et al*., 2012) screening experiment for glucuronidation activity were also done in human hepatic and intestinal microsomes. The activities of pooled human liver microsomes from 7 donors, and intestinal microsomes isolated from 10 donors were assessed for their activity toward C-1305 and C-1311 (Fig. 4). ESI-MS analysis confirmed that only one metabolite was formed from each compound by HIMs and HLMs with the retention time identical to that of the product formed with recombinant UGT1A10 (Fig. 3).

The box and whisker plots presented in Fig. 4 showed high interindividual differences among the donors in the activity of both HIM and HLM toward C-1305 and C-1311. The glucuronidation potency expressed as median HIM and HLM specific activities toward C-1305 were not significantly different, whereas median HIM activity toward of C-1311 was significantly higher than that of HLM. However, statistical comparison of the data indicates no significant differences in either median HIM or HLM activity toward C-1305 and C-1311.

Kinetic Analysis of selected recombinant UGTs, HLM and HIM with C-1305 and C-1311.

Based on activity screening data, recombinant UGT1A1, 1A7, 1A9 and 1A10 and the microsomal preparations with the highest activity, HIM34, HLM114, were subjected to kinetic analysis. The apparent kinetic constants are presented in Table 1. As indicated by screening assays, steady-state kinetics confirmed that UGT1A10 was the most active recombinant UGT isoform with V_{max} values of 2.56 and 0.63 nmol/min/mg proteins for C-1305 and C-1311, respectively. Although UGT1A10 activity (V_{max}) toward C-1305 was 4 times greater than toward C-1311, it showed a similar affinity for C-1305 and C-1311 with apparent K_m values of 94 and 71 µM, respectively. Accordingly, UGT1A10 catalytic efficiencies, measured as the $V_{max}/K_{m,}$ ratios were higher than those observed for other recombinant UGTs. The ratios were 27 µl/min/mg for C-1305, and 9 µl/min/mg for C-1311.

The lowest K_m values found were 29 µM for glucuronidation of C-1305 by UGT1A7 and of C-1311 by UGT1A1, indicating that these enzymes have the highest affinity for the glucuronidation of acridinone derivatives. Both compounds were metabolized by UGT1A9 to a moderate degree, (V_{max}

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~0.2 nmol/min/mg protein) and with similar affinities (K_m, 60 μM for both compounds). Moreover, with UGT1A9, initial velocities of both compounds increased as a function of substrate concentration to 0.15 nmol/mg protein/min at 300 μM C-1305 and to 0.14 nmol/mg protein/min at 235 μM C-1311 and then decreased (data not shown) indicating that there was substrate inhibition with high K_{si} value of 1700 μM (Table 1).

Two representative microsomal fractions from liver (HLM114) and intestine (HIM34) were selected for evaluation of kinetic parameters, for comparison to the data obtained with the recombinant enzymes. In the case of C-1305, parameters K_m and V_{max} were close for intestine and liver microsomes. However, those parameters were strongly different for C-1311. For instance, glucuronidation of this compound with HIM34 gave V_{max} significantly higher then with HLM14. On the other hand, V_{max}/K_m, values obtained for HIM and HLM for both compounds were very similar.

Glucuronidation of C-1305 and C-1311 throughout the human GI tract.

In order to extend our knowledge about the role of the GI tract in glucuronidation of C-1305 and C-1311, substrates were incubated with HIM prepared from stomach (ST), duodenum (D), jejunum (S1), intermediate small intestine (S2 and S3), ileum (S4) and colon (C) from 4 donors. The results, presented in Fig. 5, show that the two compounds were glucuronidated to different degrees by different intestinal segments and that C-1305 seemed to be a better substrate than C-1311 for UGTs distributed throughout the GI tract. Strong interindividual variations were observed in microsomes isolated from tissues throughout the length of the digestive tract. Activity was generally low in stomach and colon, as compared to the duodenum and small intestine segments.

Identification of glucuronide products.

Structural assignments for microsomal metabolites were made by comparison of ESI-MS and UV-Vis spectra of metabolites with those obtained with recombinant UGT1A10. The spectral data and the proposed structure of the C-1305 metabolite are presented in Fig. 6. The metabolite of C-1305 with m/z 514.2 [mass ion: 338.1 (C-1305)+175 (free glucuronic acid residue)+1] had a UV-Vis spectrum slightly changed from that of the substrate. Analogous observations were noticed previously with the C-1311 metabolite with m/z 526.2 [mass ion: 350.1 (C-1311)+175 (free glucuronic acid residue) +1] (Potęga *et al*., 2011). Metabolites of both C-1305 and C-1311 were sensitive to β-glucuronidase treatment (Fig. 3). To determine the functional group in C-1305 and C-1311 that is prone to

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glucuronidation, the glucuronidation activity of the recombinant UGTs and selected HIM and HLM with 8-methoxy derivatives of C-1305 and C-1311 was assayed. No glucuronidation activity was observed when 8-methoxy substrates (Cholody *et al*., 1990a, Cholody *et al*., 1992) were incubated instead of 8-hydroxy. These results strongly support the proposed metabolite structures as C-1305/C-1311 8-O-glucuronides (Fig.6).

Glucuronidation of C-1305 and C-1311 by UGT1A10 mutants and comparison to wild type UGT1A10.

To investigate the structure-function relationships of UGT1A10 in relation to the glucuronidation of C-1305 and C-1311, five recombinant proteins with point mutations of specific amino acids previously identified as being crucial for the binding of phenol substrates or the nucleotide-sugar of UDP-GlcUA were evaluated for their activity toward these substrates (Fig. 7). Changing amino acids F90 and F93 of the side chain aromatic ring to either A or L totally abolished the activity of the enzyme toward both substrates. The only substrate binding site mutant of UGT1A10 that retained activity towards the substrates was V92A; however, its specific activity was less than 35% of WT UGT1A10 with either substrate. The mutation at D393 in the N-terminal co-substrate binding domain of UGT1A10 caused total inactivation of this isoform. In contrast, in the D396A mutant, activity toward C-1305 and C-1311 was preserved at a level close to that of WT 1A10 .

Kinetic analysis of wild type and mutant UGT1A10 with C-1305 and C-1311.

The kinetic parameters of the two active UGT1A10 mutants, V92A and D396A, were determined and compared with the apparent kinetic constants of wild type isoform (Fig. 8). The activity of the V92A mutant toward C-1305 and C-1311 was decreased by 72% and 60%, respectively, relative to WT 1A10. However, increased affinity of this mutant for C-1305 (WT: K_m 94.1 μM; V92A: K_m 64.7 μM) and C-1311 (WT: 70.6 μM; V92A: 40.7 μM) was observed. The change of D396 to A produced an enzyme with a 23% decrease in the catalytic activity toward C-1305, but a 30% increase in the catalytic activity toward C-1311. For C-1305 the affinity of D396A was 2 fold higher than WT, whereas for C-1311 it remained almost unchanged as compared to WT.

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DISCUSSION

Previous studies on the *in vitro* metabolism of the antitumor acridinone derivatives, C-1305 and C-1311, demonstrated that human recombinant cytochrome P450 isoforms do not catalyze the metabolism of these compounds. In contrast, both compounds were shown to inhibit the activity of human recombinant and microsomal CYP1A2 and CYP3A4 (Potega *et al*., 2011, Fedejko-Kap *et al.,* 2011), which suggested the formation of some reactive intermediates, followed by covalent binding to the active center of the enzyme. Furthermore, the 8-hydroxyl group of C-1311 participated in peroxidase-mediated metabolism, which produced reactive intermediates susceptible to substitution in the presence of cellular nucleophiles (Mazerska *et al*., 2003).

Structural analysis of the two compounds indicated that there were two potential targets for glucuronidation, the 8-hydroxyl group and the two amino groups (Fig.1). Therefore, the current studies were undertaken to determine whether human UGTs were active toward C-1305 and C-1311 and to rigorously characterize any glucuronides formed. HPLC-MS spectra of UGT-mediated metabolic products from glucuronidation assays revealed that each acridinone were metabolized by recombinant and microsomal UGTs to a single glucuronide derivative (Fig. 3). The biosynthetic products from these experiments were treated with β-glucuronidase to verify that the products were indeed glucuronides. Protection of the 8-hydroxyl group by the addition of a methyl group eliminated glucuronidation of these compounds, confirming that these hydroxyl groups were the sole targets of the reaction and no N-glucuronidation of the two amino groups.

Several recombinant human UGTs were shown to have activity toward the C-1305 and C-1311 (Fig. 2). These studies demonstrated the involvement of UGT1A isoform; however, no activity was seen with any of the UGT2B family isoforms tested. Recombinant UGT1A1, 1A7, 1A9 and UGT1A10 were found to have activity towards these compounds. UGT1A10 expressed the highest activity, especially toward C-1305, (V_{max} values 10 times higher than those for UGT1A1, 1A7, and 1A9) (Table 1). This indicated that small differences in the structure between the two compounds (Fig.1) resulted in different interactions with UGT1A10. The compounds differ in the electron density of the triazole and imidazole rings, which has been shown to influence specific interactions of C-1305 with DNA (Lemke et al., 2004). Therefore, one can suspect that changes in the electron density of the heterocyclic ring supported by the longer distance between the amino groups in the side chain as well as by the

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presence of more easily dealkylated methyl group are responsible for C-1305 being a better substrate for UGTs

For this work, we took advantage of the availability of a unique collection of hepatic and intestinal microsomes. The intestinal samples are especially interesting due to the availability of not only microsomes derived from the full length of the small intestine, but also samples isolated from the individual segments of the GI tract. Glucuronidation activity in these human microsomes was assayed to confirm that the activities identified using recombinant UGTs were present in native human organs.

The graphs in Figs 4 and 5 representing the glucuronidation activity of microsomes derived from different human donors clearly show strong interindividual variation, particularly when microsomes from the individual segments of GI tract are assayed. This pattern is similar to that reported previously for other endogenous and exogenous compounds (Antonio *et al*., 2003, Sabolivic *et al*., 2006). UGT-mediated activity toward C-1305 and C-1311 was seen in human liver and intestinal microsomes (Table 1). The results presented in Fig. 4 demonstrated that glucuronidation of C-1311 was generally higher by HIM than by HLM ($p<0.01$), what is not observed in the case of C-1305.

Drawing clear conclusions from the kinetic analysis of the microsomal studies is nearly impossible since an unknown number of individual UGT isoforms are present in these preparations. However, the results do indicate that metabolism of C-1305 and C-1311 in both the liver and the intestine must be considered when evaluating the first pass metabolism of these drugs. The involvement of UGT1A9 which is the most highly expressed in human kidney (Harbourt *et al*., 2012,) and UGT1A7, which is expressed in the aerodigestive tract (Zheng *et al.,* 2001, 2002) in the metabolism of these compounds necessitates future investigation of the glucuronidation of these compounds in these tissues as well.

Although our main original aim was to examine the possible contribution of glucuronidation to the metabolism of these two compounds, the results steered the focus of this research to a new aspect. C-1311 and C-1305 turned out to be useful probe substrates for UGT1A10. These compound largely follow the two criteria for a good probe compound, namely that it is selective for one isoform and the individual enzyme exhibits a similar affinity for the substrate as do human tissues or microsomes (Court, 2005). The availability of several recombinant enzymes containing point mutations in the substrate and co-substrate binding sites of this isoform allowed for additional structure function relationship studies using these compounds. Therefore, we were able to test the hypothesis that the

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F90, F93, V92 and D393, D396 amino acids of UGT1A10 are important for proper recognition and conjugation of C-1311 and C-1305.

These studies demonstrated that F90 is critical and F93 is significant for glucuronidation of both compounds (Fig. 7) The importance of F90 and F93 has also been shown to be crucial for the glucuronidation of p-nitrophenol and 4-methylumbelliferone (Xiong *et al*., 2006), dopamine (Itäaho *et al*., 2009), estrogen (Starlard-Davenport *et al*., 2007, Höglung *et al*., 2011) and warfarin (Miller *et al*., 2008). As with the hydroxylated estrogens, 6- and 7-hydroxylated warfarin and dopamine glucuronidation, the mutation of F90 to A totally abolished UGT1A10 activity toward C-1305 and C-1311. Furthermore, in contrast to other reports, the F90L mutation was also destructive for UGT1A10 activity. Interestingly, the V_{max} values found with the V92A mutation and C-1305 and C-1311 were reduced 3.5 to 2.5 fold, respectively, whereas this mutation had preserved or even increased activity of UGT1A10 toward estrogens and warfarin metabolites (Miller *et al*., 2008, Höglung *et al*., 2011).

Additional experiments were done to clarify the effect of the D393 and D396 mutations localized in the GlcUA binding site on the catalytic activity of UGT1A10. Of the two mutated residues, D393 is apparently essential for binding UDP-GlcUA because of the complete loss of activity in the D393A mutant. In contrast, D396 plays a minor role in substrate turnover. Therefore, our results have confirmed the crucial role of D393 for UGT catalysis and indicated a slight influence of the D396A mutation on the UDP-GlcUA association for C-1305 and C-1311 in the catalytic center of UGT1A10. The novelty of the role of D393 in UGT1A10 activity has broader implications because of the conservation of this residue in all human UGTs (Xiong, et al. 2008).

It is increasingly recognized that UGTs are involved in cancer cell drug resistance (Cummings *et al*., 2003, Kostrubsky *et al.,* 2005, Gagnon *et al*., 2006, Tallman *et al*., 2007, Starlard-Davenport *et al.,* 2010, Lazarus and Sun, 2010, de Almargro *et al.,* 2011). It is also recognized that glucuronidation does not always result in inactivation of biologically active compounds, but can produce products that retain or have significantly increased biological activity (Ritter 2000, Sallustio *et al.,* 2006). The best examples of this are morphine (Osborne R *et al*.,1992, Klepstad P *et al*., 2000, Murthy *et al.*, 2002) as well as retinoids and estrogens, metabolites of which all appear to exhibit biological effects distinct from the parent compounds (Ritter, *et al.* 2000). In parallel studies (manuscript in revision), we have been able to show that C-1305 represents another example of this phenomenon. We have been able

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to show that, in cancer cell models, the C-1305 glucuronide has cytotoxic properties that are greater than those for the native compound. The present studies are essential also because they identify the UGT isoforms involved in this substrate activation.

In summary, the present studies have demonstrated that both the drugs investigated here are glucuronidated by several UGTs expressed in the liver, intestine, kidney, and the aerodigestive tract. The information on which UGT isoforms are involved in the glucuronidation of C-1305 and C-1311 is very important for the design of new drugs and the management of existing ones. In addition, the knowledge gained from these structure function experiments could be exploited for the design of analogs of clinical and pharmacological importance with the aim of increasing and/or decreasing the biological response of UGTs and/or eliminating any undesired side effects of glucuronidation. The question of whether the glucuronidation contributes to overall drug toxicity or drug resistance in certain tissue specific cancers has yet to be answered.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Mazerska, Radominska-Pandya, Fedejko-Kap

Conducted experiments: Fedejko-Kap, Bratton

Contributed new reagents or analytical tools: Radominska-Pandya, Finel

Performed data analysis: Fedejko-Kap, Mazerska, Radominska-Pandya, Bratton, Finel,

Contributed to the writing: Fedejko-Kap, Mazerska, Radominska-Pandya, Bratton, Finel,

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Footnotes

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LEGENDS FOR FIGURES

Figure 1.

Structures of the triazoloacridinone, C-1305, and the imidazoacridinone, C-1311.

Figure 2.

Glucuronidation of C-1305 (**A**) and C-1311 (**B**) by human recombinant UGTs. Glucuronidation activities of human recombinant UGTs (5 µg protein) UGT1A1, 1A3, 1A4, 1A6, 1A7-1A10, 2B4, 2B7, 2B15 and 2B17 were measured. The substrate and co-substrate (UDP-GlcUA) concentrations were 0.1, 0.2 and 3 mM, for C1305, C1211 and UDP-GlcUA, respectively, and the reactions were incubated for 60 minutes. Activities are expressed in nmol/mg protein/min and shown with standard deviations based on 3 experiments. *** $p<0.001$, indicates the significant difference of 1A10 activities in comparison to all other UGTs (one-way ANOVA)

Figure 3.

Metabolism of C-1305 (**A**) and C-1311 (**B**) by human recombinant UGT1A10. Representative HPLC chromatograms are shown from 60 min incubations of 5 µg UGT1A10 (solid line), 5 µg UGT1A10 and 1000 U β-glucuronidase, GUS (dotted line) with 0.2 mM substrate and 3 mM UDP-GlcUA and control with enzymes and cofactors but without C-1305 (dashed-dotted line),

Figure 4.

Glucuronidation of C-1305 and C-1311 by human intestinal and liver microsomes. Glucuronidation activities of HIM and HLM were measured using microsomes isolated from different donors (20 µg total protein). The substrates concentration was 0.2 mM, the UDP-GlcUA concentration was 3 mM and the reactions were incubated for 60 minutes. For each box and whisker plot, the line bisecting the box is the median value, the upper and lower limits of the box are the 25th and 75th percentile values, and the error bars (whiskers) extend to the lowest and highest values. Statistical comparisons, performed using the Kruskal-Wallis nonparametric ANOVA followed by Dunn's multiple comparison test, are indicated by the lines above the plots. **, $p < 0.01$; ns, not significant.

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Figure 5.

Glucuronidation of C-1305 (**A**) and C-1311 (**B**) by human stomach and intestine microsomes. Microsomes (20 μg) were prepared from the mucosa of stomach (ST), duodenum (D), four segments of small intestine (S-1 to S-4, proximal to distal), and colon (C) from four different donors. The substrate and UDP-GlcUA concentrations were 0.1 and 3 mM, respectively, and the reactions were incubated for 60 minutes. For each box and whisker plot, the line bisecting the box is the median value, the upper and lower limits of the box are the 25th and 75th percentile values, and the error bars (whiskers) extend to the lowest and highest values. ***, $p < 0.001$, indicates a significant effect of donor and fragment with significant interaction (two-way ANOVA with Benferoni post test).

Figure 6.

Structure analysis of metabolite M1 of C-1305 from incubations of microsomal proteins and UGT1A10. ESI-MS and UV-Vis spectra of metabolite and parent compound were extracted from the appropriate HPLC samples.

Figure 7.

Glucuronidation activities of human recombinant wild type UGT1A10 and its mutants toward C-1305 (**A**) and C-1311 (**B**). UGT1A10 and its mutants were assayed by incubating membrane fractions of recombinant UGTs with substrate (0.1 and 0.2 mM for C-1305 and C-1311, respectively) and UDP-GlcUA (3 mM) for 60 min at 37°C. Activities are expressed in nmol/mg total protein/min and shown as mean ± SD for 3 determinations. Levels were considered significant at $p < 0.001$. * Indicates significance of results with respect to WT 1A10 at the concentration of 0.1 mM; • indicates significance of results with respect to WT 1A10 at the concentration of 0.2 mM. Analysis of variance (two-way ANOVA with Bonferroni post hoc analysis) was carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Fig. 8.

Kinetic constants for C-1305 (A) and C-1311 (B) glucuronidation by recombinant WT UGT1A10 and mutants. Glucuronidation activities of recombinant WT UGT1A10 (●) and the D396A (▲) and V92A (■) mutants were measured by incubating membrane fractions containing recombinant UGT1A10 and its mutants (5 μg) with increasing concentrations (shown in the figure) of the substrates at a constant concentration of UDP-GlcUA (3 mM) for 60 min at 37°C. Curve fits and kinetic constants were

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determined using GraphPad Prism 4 software. The graphical fits of the data from each of the analyses with each substrate (mean ± SD of 3 determinations) are shown. Km values are given in μM, and V_{max} values are given in nmol/mg protein/min.

Table 1. Steady-state parameters for glucuronidation of C-1305 and C-1311 by UGT isoforms, HIM and HLM.

Parameters were determined from the fit of initial velocities to a Michaelis-Menten kinetic scheme or substrate inhibition equations using GraphPad Prism 4. (M-M:Michaelis-Menten; SI: Substrate inhibition; Hill: Hill equation). Data are presented as mean ± SEM of three determinations.

Enzyme	Kinetic model	Substrate							
		C-1305				C-1311			
		K_m (µM)	V_{max} (nmol/min/ mg protein)	K_{si} (µM)	V/K (µl/min/mg)	K_m (µM)	V_{max} (nmol/min/ mg protein)	K_{si} (µM)	V/K (µl/min/mg)
UGT1A1	M-M	149 ± 29	0.262 ± 0.017	-	1.8	28.9 ± 9.0	0.115 ± 0.008	-	4
UGT1A7	M-M	29.3 ± 3.2	0.306 ± 0.008	-	11	N.D.	N.D.	-	-
UGT1A9	SI	54.6 ± 22.4	0.201 ± 0.038	1651 ± 1106	3.7	63.3 ± 22.6	0.206 ± 0.037	958 ± 452	3.3
UGT1A10	M-M	94.1 ± 10.3	2.557 ± 0.082	-	27	70.6 ± 6.01	0.624 ± 0.015	-	8.8
HIM 34	M-M/Hill[#]	76.9 ± 11.1	0.413 ± 0.015	-	5.4	158.6 ± 5.2	0.823 ± 0.013[#]	-	5.2
HLM 114	M-M	71.3 ± 6.0	0.386 ± 0.009	-	5.4	39.3 ± 6.1	0.156 ± 0.006	-	4
		ns	ns			***	***		

[#], Hill equation; n = 1.65

ns, not significant (p>0.05); ***, p<0.001. Values were compared with Student's unpaired t test.

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Figure 1

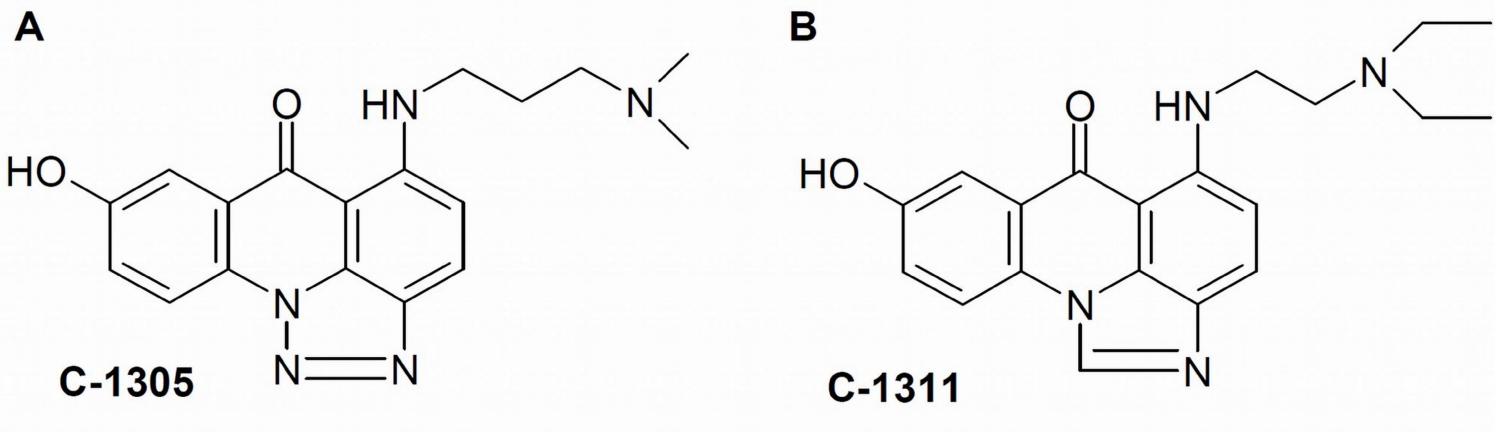

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Figure 2

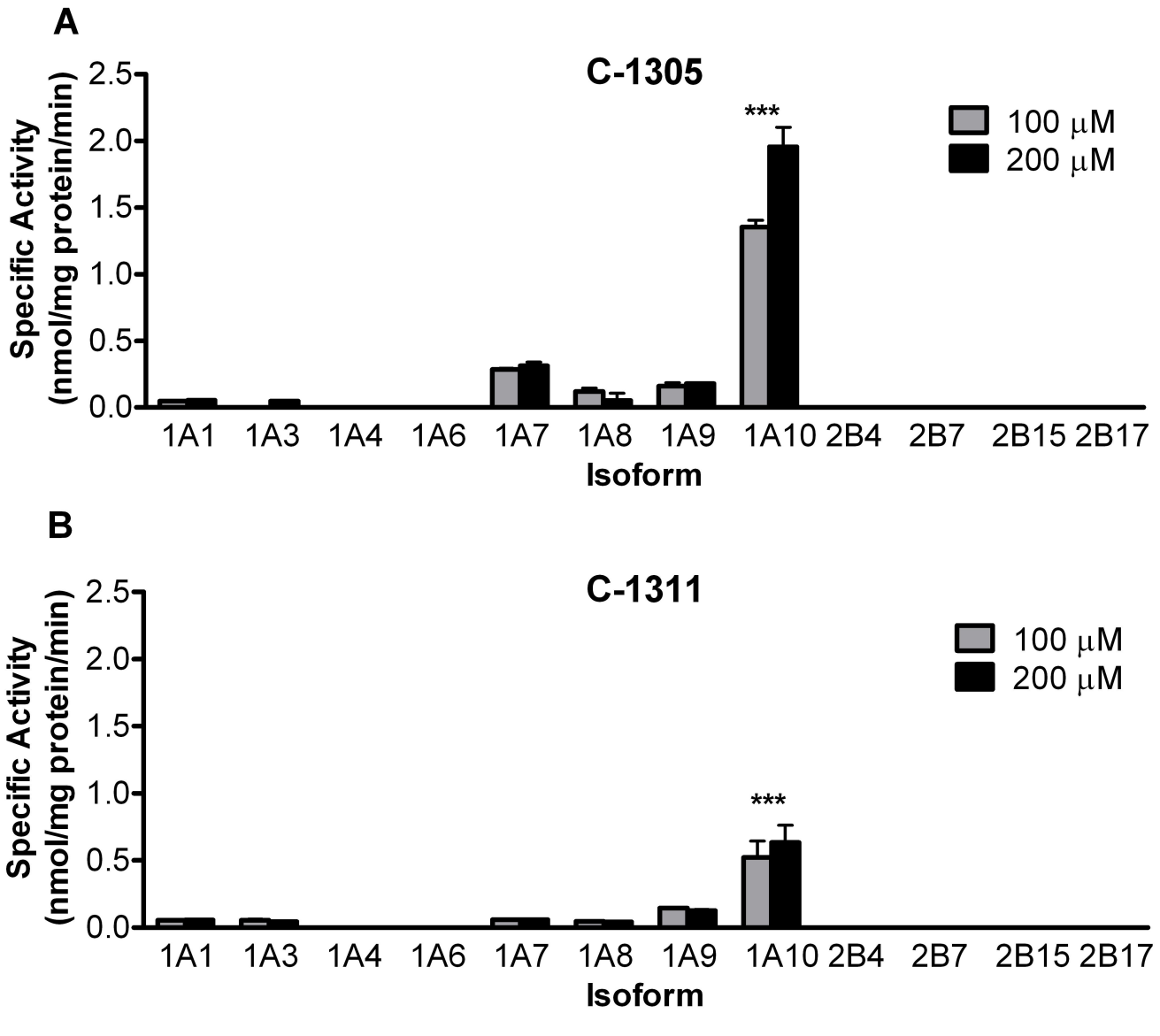

Figure 3

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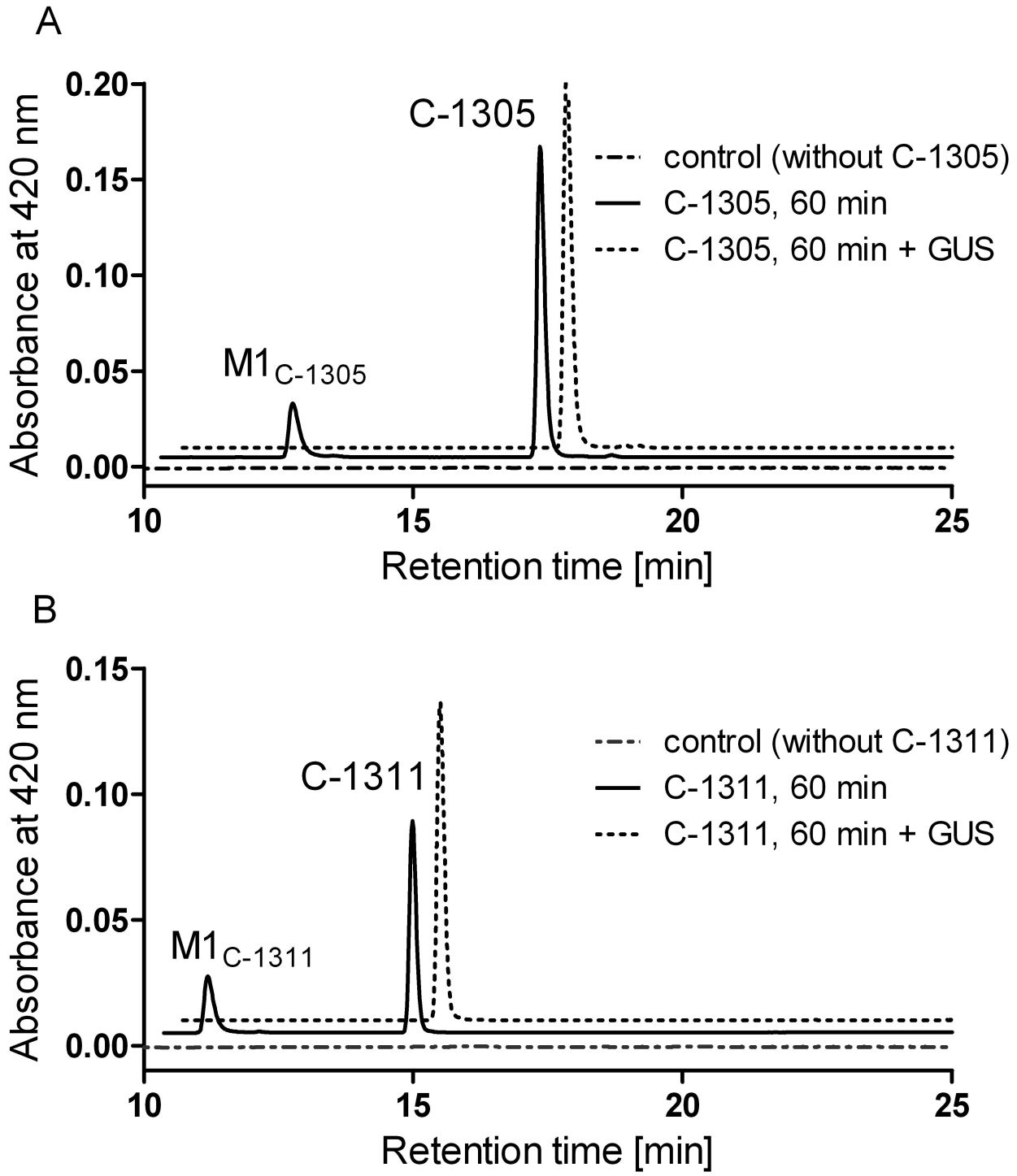

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Figure 4

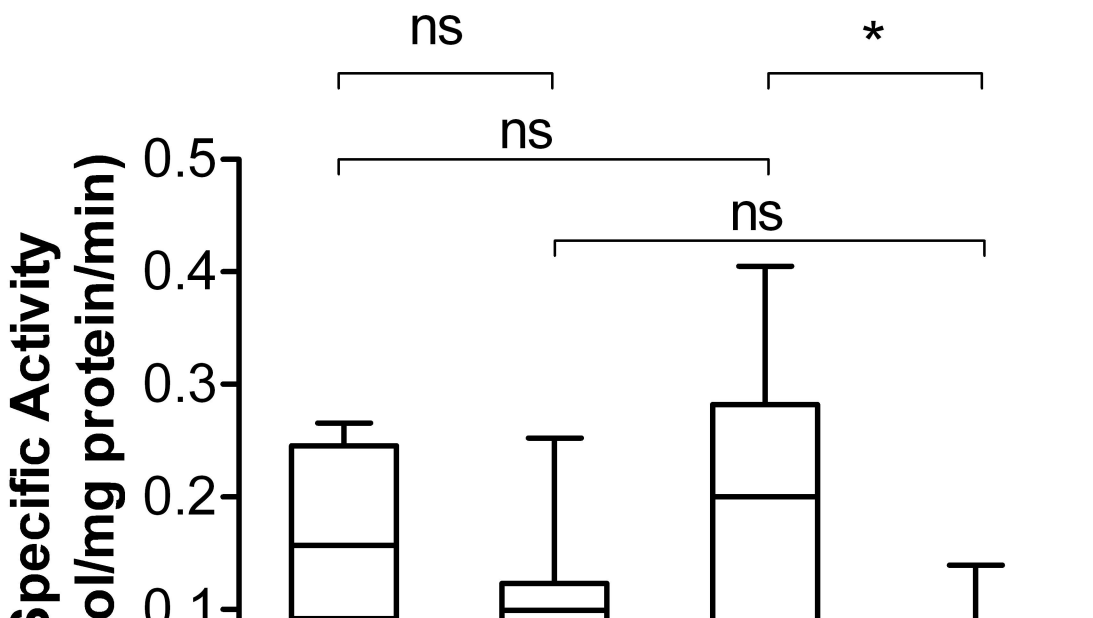

Figure 5

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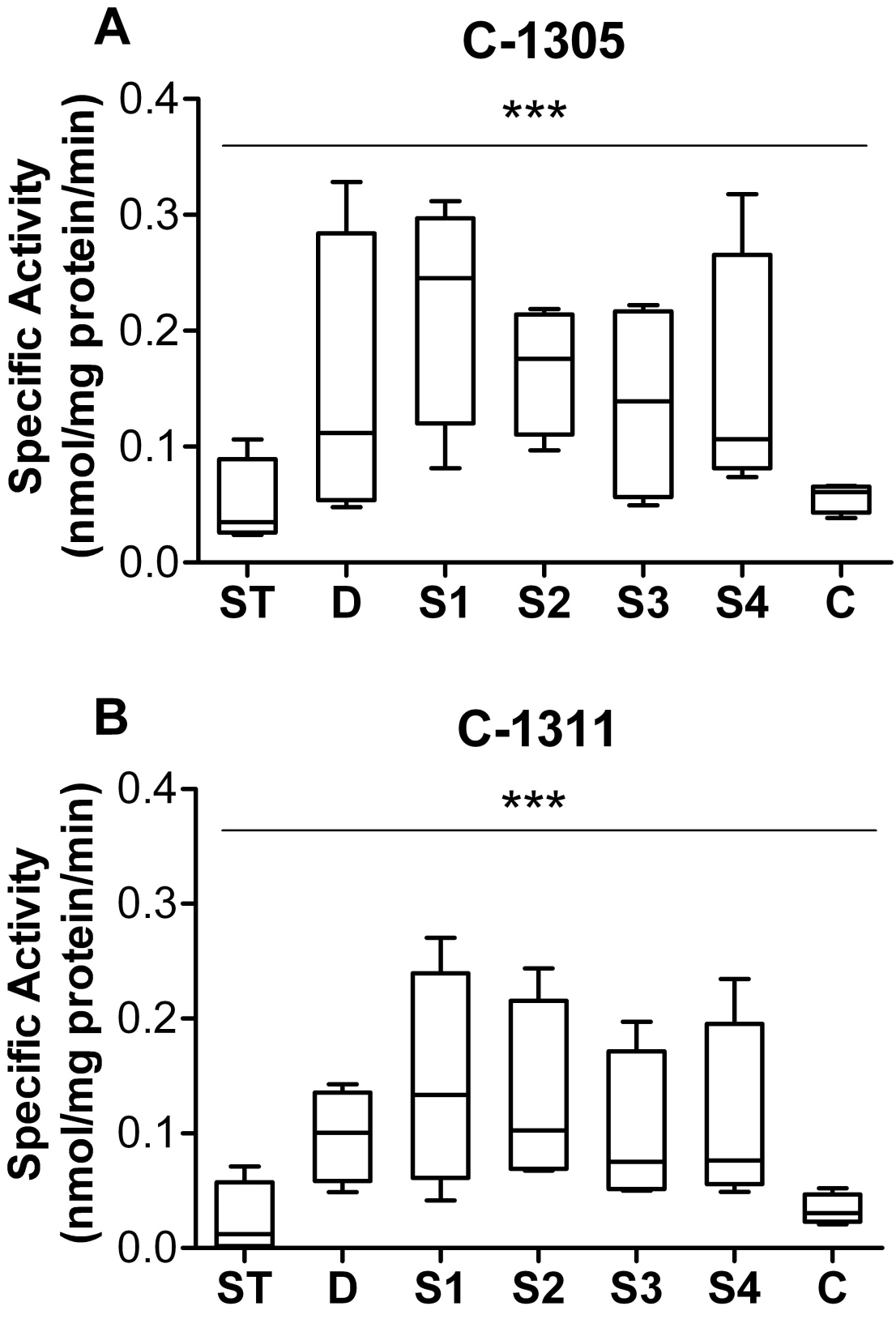

Figure 6

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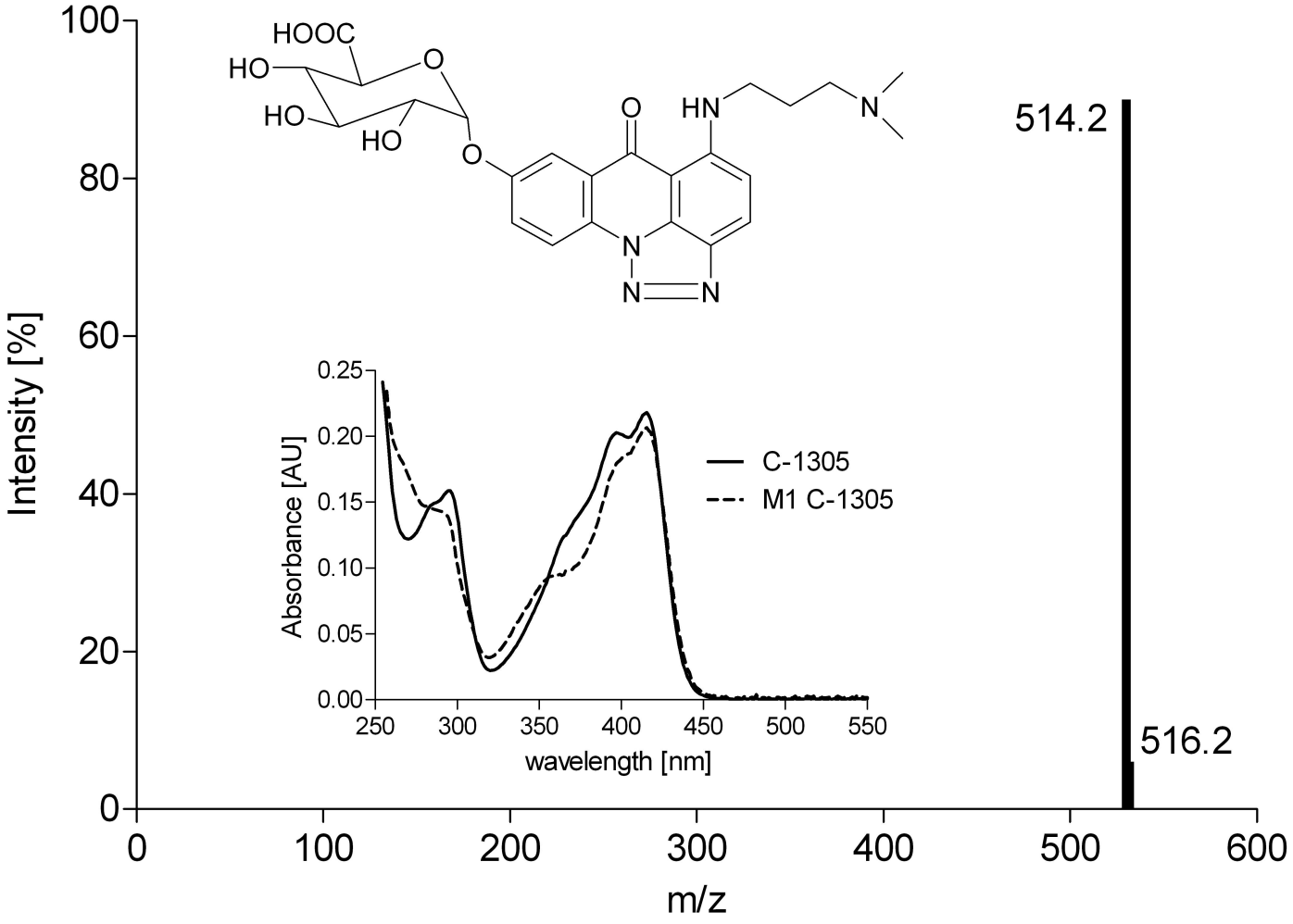

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Figure 7

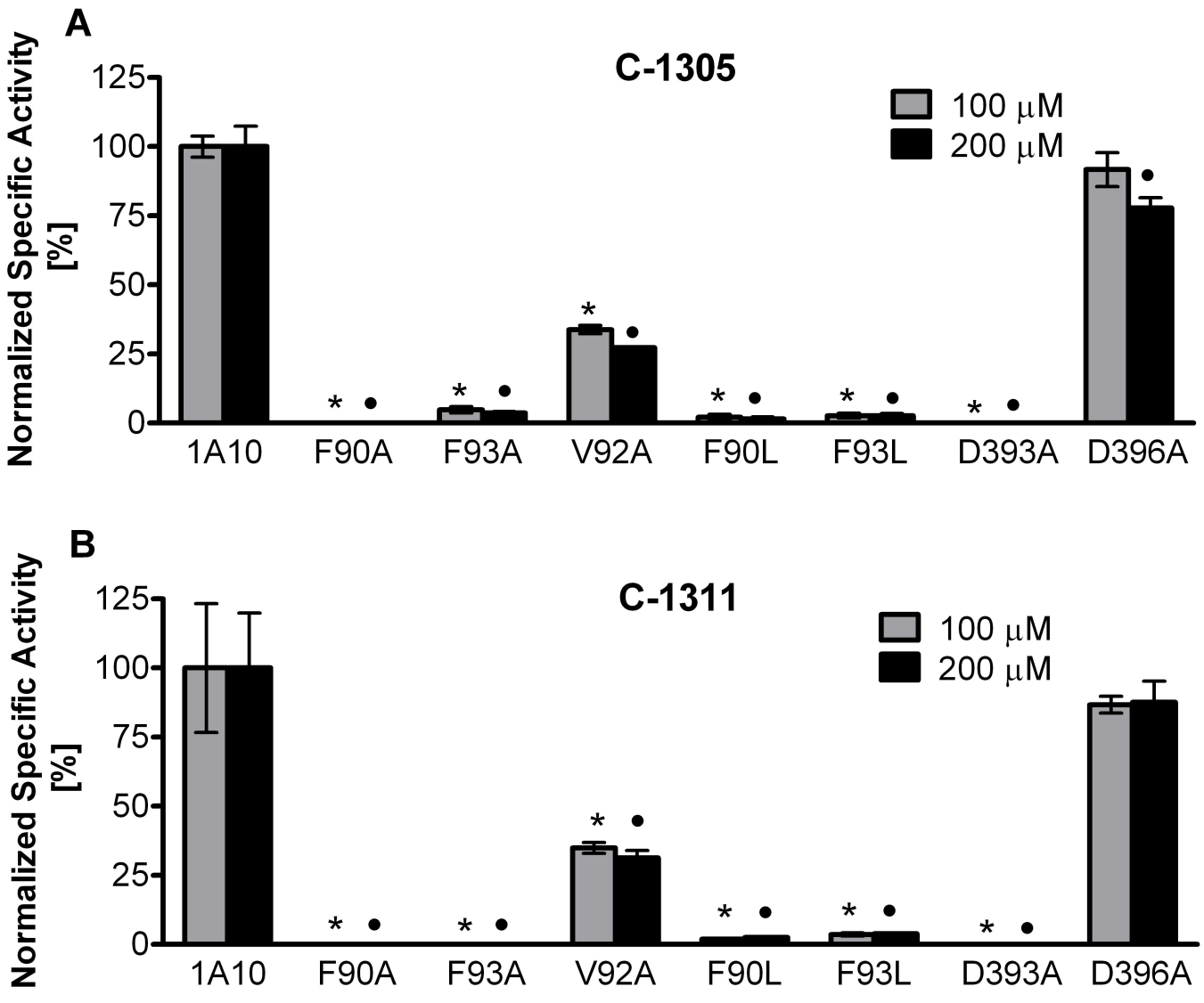

Figure 8

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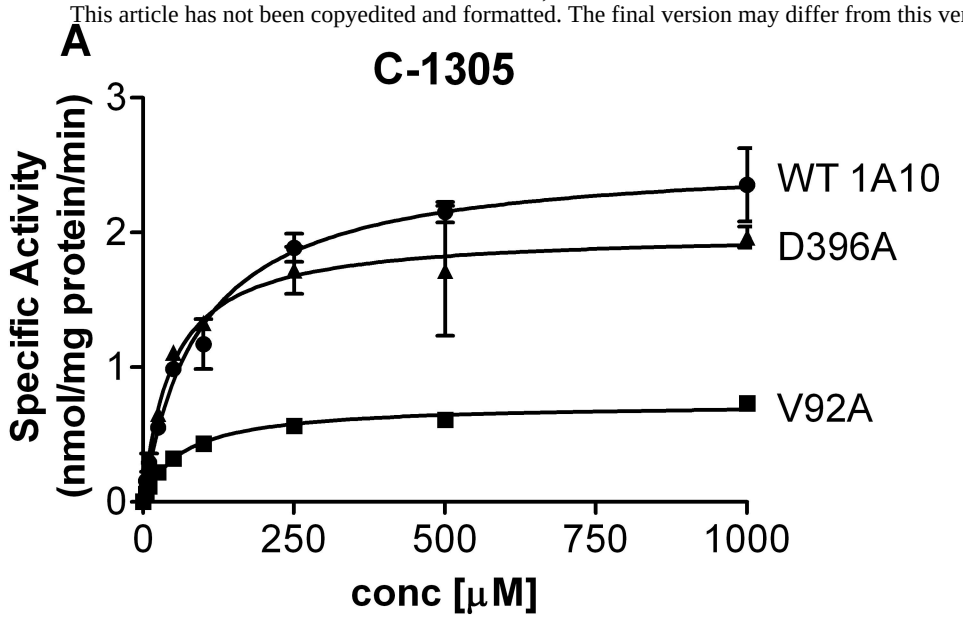
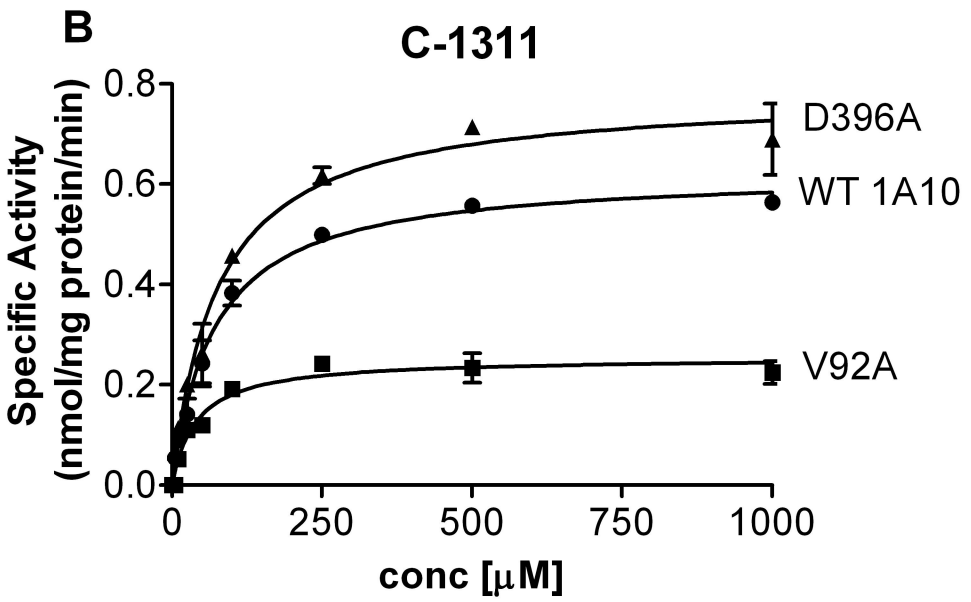


UGT isoform	K_m	V_{max}
1A10	94.1 ± 10.3	2.56 ± 0.08
D396A	47.6 ± 7.56	1.99 ± 0.08
V92A	64.7 ± 5.60	0.73 ± 0.02



UGT isoform	K_m	V_{max}
1A10	70.6 ± 6.01	0.62 ± 0.01
D396A	75.4 ± 8.48	0.78 ± 0.02
V92A	40.7 ± 6.93	0.25 ± 0.01