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An assessment of the *in vitro* inhibition of cytochrome P450 enzymes (CYP), UDP-glucuronsyltransferases (UGT) and transporters by phosphodiester- or phosphorothioate-linked oligonucleotides

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Abbreviations used are: Ac, beta-actin; ASO, antisense oligonucleotide; BCRP, breast cancer resistance protein; CHH, Cryopreserved Human Hepatocytes; DDI, Drug-drug interaction; EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; GP; Glutathione peroxidase; HLM, human liver microsomes; IC_{50}, inhibitor concentration that causes 50% inhibition; LC-MS-MS, liquid chromatography/tandem mass spectrometry; MATE, multidrug and toxin extrusion; MDI, metabolism-dependent inhibition; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; OCT; organic cation transporter; PBS, phosphate buffered saline; PD, phosphodiester; P-gp, P-glycoprotein; PS, phosphorothioate; TDI, time-dependent inhibition; UGT, UDP-glucuronosyltransferase.

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ABSTRACT

Oligonucleotides represent an expanding class of pharmacotherapeutics in development for various indications. Typically, oligonucleotides are developed with phosphorothioate linkages for the improvement of biological stability; however limited data are available on the potential of these molecules to cause drug-drug interactions (DDIs). In the present study, two non-therapeutic oligonucleotides with either phosphodiester (PD-GP and PD-Ac) or phosphorothioate (PT-GP and PT-Ac) linkages were evaluated in vitro for their potential to inhibit P450s and UGTs in both human liver microsomes (HLM) and cryopreserved human hepatocytes (CHH) and to inhibit select transporters in expression systems. PD-GP and PD-Ac had little-to-no inhibitory effect on any P450 or UGT enzymes in HLM and CHH except for PD-Ac in HLM for CYP2C19 (IC_{50} = 29 μM). Conversely, PT-GP and PT-Ac caused direct inhibition of almost all P450 and UGT enzymes, with CYP1A2 (IC_{50} values 0.8-4.2 μM), CYP2C8 (IC_{50} values 1.1-12 μM) and UGT1A1 (IC_{50} values 4.5-5.4 μM) inhibited to the greatest extent. There was evidence of possible time-dependent inhibition (TDI) of CYP enzymes with PT-GP and PT-Ac for CYP2B6, CYP2C8, CYP2C19, CYP2C9, CYP2D6 and CYP3A4/5; however, this TDI was reversible. In contrast to HLM, there was little-to-no direct CYP inhibition by any oligonucleotide in CHH (except for PD-Ac with CYP2C19 [IC_{50} = 36 μM] and TDI by PT-GP with CYP2C8), demonstrating test system-dependent outcomes. Inhibition was observed for the organic anion uptake transporters OATP1B1, OATP1B3, OAT1, OAT3, and OCT2 (IC_{50} values 12-29 μM) but not OCT1 or the efflux transporters BCRP and P-gp by the phosphorothioate oligonucleotides.

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INTRODUCTION

Oligonucleotide-based therapies represent an intriguing class of molecules being developed for a range of indications such as oncology, metabolic and genetic disorders and infectious diseases (Aartsma-Rus, 2016; Vasko et al., 2017; Rossor et al., 2018). Typically, these molecules are developed as small complementary base pair sequences to targets, known as anti-sense oligonucleotides (ASOs), allowing for specific knockdown of gene expression through Watson-Crick base pair binding with target mRNA followed by digestion by cellular nucleases. ASOs are often chemically modified to improve their pharmacokinetic properties such as phosphorothioate linkage in lieu of phosphodiester linkage to improve biological stability (see Figure 1), as well as other modifications to improve absorption and localization to the target (Yu et al., 2013; Chen et al., 2018). Generally, phosphorothioate based ASOs have high plasma protein binding (>90%), rapidly distribute to tissues, while being subject to slow metabolism by cellular nucleases and eventual clearance primarily through urinary excretion of parent molecule and fragment ASO metabolites (Geary, 2009).

There have been limited studies on the potential for ASOs to act as perpetrators of pharmacokinetic drug-drug interactions (DDIs), however these reports to date have shown no clinical DDIs with an array of co-medications cleared through a multitude of clearance pathways (Adjei et al., 2003; Villalona-Calero et al., 2004; Geary et al., 2006; Yu et al., 2009; Li et al., 2014). These include clinical interaction studies with warfarin (CYP2C9/3A4), simvastatin (CYP3A4), ezetimibe (multiple UGTs), rosiglitazone (CYP2C8/2C9), glipizide (CYP2C8/2C9), metformin (renal), cisplatin (renal) and gemcitabine (nucleoside kinases). As ASOs are typically large (>5 kDa) polyanionic molecules and given their non-P450 dependent metabolism and clearance, these molecules would not be expected to inhibit drug metabolizing enzymes.

The recent 2017 FDA draft industry guidance on *In vitro metabolism- and transporter-mediated drug-drug interaction studies*

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(https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM581965.pdf) and the 2013 *EMA Guideline on the investigation of drug interactions* (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf) provides guidelines on the specific types of in vitro studies necessary for new drug applications. While neither the FDA nor EMA guidance documents have specific recommendations for ASO DDI evaluation, these molecules do fall under the scope of overall drug-drug interaction testing despite their distinct physicochemical properties. As a result, studies such as inhibition of P450s and UDP-glucuronosyltransferases (UGTs) are required to be performed, including evaluations of time-dependent inhibition (TDI) for P450 enzymes. Typically, assessments of both TDI (pre-incubation of perpetrator compound) and metabolism-dependent inhibition (MDI; preincubation of compound with NADPH co-factor) are performed in the industry (Parkinson et al., 2011), as the former simply measures the effect of time on enzyme activity loss while the latter assess the impact of P450 metabolism on enzyme activity loss. The recent FDA and EMA recommendations also specify the use of subcellular fractions as well as cell-based systems, such as human liver microsomes (HLM) and cryopreserved human hepatocytes (CHH), for these studies. Furthermore, the evaluation of uptake and efflux transporter inhibition is recommended to be examined with appropriate test systems.

Upon performing the aforementioned in vitro studies in our laboratory with proprietary phosphorothioate ASO molecules, we unexpectedly observed P450 inhibition in human liver microsomes (HLM). We hypothesized that this was not oligonucleotide sequence specific inhibition, but rather an artifact due to the presence of the phosphorothioate linkage. This observation prompted us to perform the present study with two generic non-therapeutic oligonucleotide sequences (partial sequences to glutathione peroxidase and beta-actin) with and without phosphorothioate linkages as surrogates for therapeutic ASOs. In the present study, phosphorothioate- and phosphodiester-linked oligonucleotides were evaluated as P450

and UGT inhibitors in both HLM and CHH. Furthermore, the potential for select transporter inhibition was also evaluated, specifically the uptake transporters such as organic anion transporting polypeptides (OATP), organic anion transporters (OAT), organic cation transporters (OCT) and the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). Overall this study reconciles in vitro DDI findings with the lack of interaction in clinical DDI reports for the oligonucleotide biomolecule class.

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MATERIALS AND METHODS

Oligonucleotides

Two ~20 base oligonucleotides with partial sequences towards glutathione peroxidase (5′-GCTCGTTCATCTGGGTGTAGT-3′) and beta actin (5′-CCTCCTCTTTGTTCCCTTCT-3′), each with either a phosphodiester or phosphorothioate linkage (generic structures shown in Figure 1) were purchased from Integrated DNA Technologies (Coralville, IA).

Chemicals and Reagents

Chenodeoxycholic acid (CDCA), digoxin, estradiol glucuronide, estrone sulfate, morphine, 1-naphthol, saccharic acid 1,4-lactone, oxazepam, *p*-aminohippuric acid, prazosin, propofol, testosterone and trifluoperazine were purchased from Sigma-Aldrich (St. Louis, MO). Levomedetomidine was a gift from Orion Corporation (Espoo, Finland). [^{3}H]-Estradiol glucuronide and [^{3}H]-Estrone sulfate were purchased from Perkin Elmer (Waltham, MA). [^{14}C]-Metformin was purchased from Moravek Biochemicals (Brea, CA). [^{14}C]-Tetraethylammonium bromide and [^{3}H]-*p*-aminohippuric acid were purchased from American Radio-labeled Chemicals (St. Louis, MO). d_5-Testosterone 17-*O*-glucuronide, d_5-oxazepam-*N*-glucuronide and prochlorperazine glucuronide used as internal standards were prepared at Sekisui XenoTech, LLC (Kansas City, KS). All other deuterated glucuronides were purchased from Toronto Research Chemicals (Toronto, Canada). The sources of all other reagents have been described previously (Parkinson et al., 2011; Kazmi et al., 2014; Kazmi et al., 2015b; Vermeer et al., 2016).

Test systems

Pooled human liver microsomes (HLM, n = 200, mixed gender) and pooled cryopreserved human hepatocytes (CHH, n = 100, mixed gender) were prepared from non-transplantable

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livers and characterized at Sekisui XenoTech, LLC (Kansas City, KS) as described previously (Pearce et al., 1996; Parkinson et al., 2004). HEK293 (Human embryonic kidney 293) cells expressing transporter (HEK293 cells transfected with vectors containing human transporter cDNA for OATP1B1, OATP1B3, OAT1, OAT3 and OCT2) and control cells (HEK293 cells transfected with only vectors) were supplied by the American Type Culture Collection (Manassas, VA) and transfected with the vector containing transporter cDNA by ADME & Tox. Research Institute, Sekisui Medical Co., Ltd. (Tokai, Japan); MDCKII cells transfected with BCRP were obtained from Netherlands Cancer Institute (Amsterdam, Netherlands); and Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA).

In vitro P450 and UGT inhibition

The effects of oligonucleotides on P450 enzymes in HLM were evaluated (n=2) in IC_{50} experiments with and without a 30 min preincubation step (in the presence and absence of NADPH) as described previously (Parkinson et al., 2011). For CHH, incubations were conducted in duplicate 100 µL mixtures containing 0.5 million cells/mL in Krebs-Henseleit buffer (KHB). Reactions were initiated by the addition of CHH to the oligonucleotides and incubations were conducted at 37°C with 95% humidity and 5/95% CO_2/air for 0, 30 and 90 min, followed by addition of a P450 marker substrate (or the broad specificity UGT substrate 4-methylumbelliferone; 4-MU) at a concentration approximately equal to its K_m (see Table 1). For incubations with sonicated hepatocytes (conducted with 10 µM PT oligonucleotides only), CHHs were disrupted with a probe sonicator (45 seconds at 40%-60% amplitude). Reactions were terminated after 10 min by the addition of 100 µL of acetonitrile containing the appropriate internal standard. Precipitated protein was removed by centrifugation (920 RCF for 10 min at 10°C) followed by LC-MS/MS analysis. To assess whether P450 inhibition was caused by a low molecular weight contaminant, the oligonucleotides (100 µM) were filtered through a 3 kDa micro-centrifuge filter (Millipore, Billerica, MA) with the filtrate (n=2) assessed for P450 inhibition

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as described above. The reversibility of P450 time-dependent inhibition (TDI) was evaluated as described previously (Ogilvie et al., 2011). Briefly, a 30-min pre-incubation of the phosphorothioate oligonucleotides (10 μM) in HLM was performed in duplicate, followed by ultracentrifugation of microsomes (100,000 x g; 60 min, 4°C). The supernatant fraction was discarded and the microsomal pellet was rinsed with potassium phosphate buffer (50 mM, pH 7.4) followed by resuspension in 250 mM sucrose. Protein concentration was determined by the Pierce BCA Protein Assay (Pierce, Rockford IL) and residual P450 activity was assessed as described above. For UGT inhibition, assays were conducted as described previously (Kazmi et al., 2014; Kazmi et al., 2015b). Briefly, oligonucleotides were incubated in duplicate at 37°C in 150 μL incubation mixtures containing pooled HLMs (≤ 0.1 mg/mL), Tris buffer (100 mM, pH 7.7), $MgCl_2$ (10 mM), EDTA (1 mM, pH 7.4), saccharic acid 1,4-lactone (0.1 mM), UDP-GlcUA (10 mM), and a UGT marker substrate at a concentration approximately equal to its K_m, at the final concentrations indicated in Table 1 as described previously. Reactions were initiated by the addition of UDP-GlcUA and terminated after 5 or 10 min by the addition of 175 μL of acetonitrile containing the appropriate internal standard. Precipitated protein was removed by centrifugation (920 RCF for 10 min at 10°C) followed by LC-MS/MS analysis. TDI for UGT enzymes was assessed with a single concentration of each oligonucleotide (30 μM) with and without a 30 min preincubation step (in the presence and absence of UDP-GlcUA) with the methodology described above.

In vitro uptake and efflux transporter inhibition

To evaluate inhibition of uptake transporters, HEK293 cells expressing various human uptake transporters were plated in 24-well plates and grown in an incubator (37°C, 95% relative humidity, 5% CO_2) for two days. On the day of the assay, cells were pre-incubated with oligonucleotides or solvent control (n=2) for 15 min followed by a 2-min incubation with a combination of probe substrate (a mixture of cold and radiolabeled compound) and

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oligonucleotide. 50 nM estradiol 17-β-glucuronide substrate was used for OATP1B1 and OATP1B3 assays (with approximate equimolar radiolabeled and cold substrate); 1 μM *p*-aminohippuric acid for OAT1 (1:4 ratio of radiolabeled to cold substrate); 50 nM estrone 3-sulfate for OAT3 (approximately equimolar radiolabeled and cold substrate); and 5 μM tetraethylammonium bromide and 10 μM metformin for OCT1 and OCT2 respectively (all radiolabeled). Uptake assays were terminated by aspiration of the incubation media followed by washing the cells three times, first a rinse with ice cold PBS containing 0.2% BSA then two rinses with ice cold PBS. For analysis, cells were extracted with 0.1N NaOH for scintillation counting on a Microbeta^2 instrument (Perkin Elmer, Waltham, MA). Evaluation of efflux transporter inhibition was conducted in Caco-2 (MDR1/P-gp) or MDCK-II cells (transfected with BCRP) plated on 24-well trans-well plates. The bidirectional permeability of specific probe substrates (10 μM digoxin for Caco-2 cells and 1 μM prazosin for MDCK-II cells) was measured (n=2) in the presence of oligonucleotides. Oligonucleotides were added to both apical and basolateral sides for 120 min. After the incubation, samples were stopped with 50:50 v/v methanol:water and analyzed by LC-MS/MS.

Analytical methods

Analysis of all P450 and UGT metabolites was conducted by LC-MS/MS as shown in Table 1 and described previously (Parkinson et al., 2011; Kazmi et al., 2014; Kazmi et al., 2015b). The analytical methods used to quantify digoxin and prazosin are described as follows. Analyst Instrument Control and Data Processing Software (AB SCIEX, version 1.6.1) was used to analyze digoxin and prazosin for data collection and integration, including calibration standards. For the analysis of digoxin, an AB SCIEX 5500 QTrap mass spectrometer in positive mode (5500 V) was employed in tandem with Phenomenex Gemini NX (C18, 3 μM, 50 x 2.0 mm, Torrance, CA) column (at 40°C) and Luna C8 Guard Column (4.0 x 2.0 mm) for separation in a

mobile phase of 0.2% formic acid in water and 0.2% formic acid in acetonitrile and an injection volume of 0.5 µL. Mobile phase flow rate was 0.8 mL/min, and the mass transitions used to identify digoxin were *m/z* = 781.4 and 651.4. Deuterated digoxin was used as an internal standard and identified with mass transitions of *m/z* = 784.5 and 654.5. For the analysis of prazosin, an AB SCIEX 5500 QTrap mass spectrometer in positive mode (3500 V) was employed in tandem with a Waters Atlantis (dC18, 5 µM, 100 x 2.1 mm, Milford, MA) column (at 40°C) and Luna C8 Guard Column (4.0 x 2.0 mm) for separation in a mobile phase of 0.2% formic acid in water and 0.2% formic acid in acetonitrile and an injection volume of 1 µL. Mobile phase flow rate was 0.7 ml/min, and the mass transitions used to identify prazosin were *m/z* = 384.0 and 95.0. Deuterated prazosin was used as an internal standard and identified with mass transitions of *m/z* = 392.0 and 95.0.

Statistical analyses

The means of all replicates were used in all calculations. All IC_{50} values were determined with GraFit (version 7.0.2; Erithricus Software Ltd., Surrey, UK). All other data were processed with Microsoft Excel 2010 (Microsoft, Redmond, WA). All of the aforementioned experiments were performed in accordance with Sekisui XenoTech standard operating procedures (SOPs) in the spirit of Good Laboratory Practices (GLP).

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RESULTS

Inhibition of P450 and UGT enzymes by oligonucleotides in human liver microsomes. To evaluate both phosphodiester- and phosphorothioate-linked oligonucleotides as inhibitors of P450 and UGT enzymes in HLM, 0.1-100 μM of either phosphodiester glutathione peroxidase oligonucleotide (PD-GP), phosphorothioate glutathione peroxidase oligonucleotide (PT-GP), phosphodiester beta actin oligonucleotide (PD-Ac) and phosphorothioate beta actin oligonucleotide (PT-Ac) was incubated with HLM (≤ 0.1 mg/mL) in the presence or absence of cofactor and with or without a pre-incubation step as described in *Materials and Methods*. As shown in Table 2, the oligonucleotides containing phosphodiester linkages (PD-GP and PD-Ac) had little-to-no inhibitory effect on any of the P450 and UGT enzymes in HLM with IC_{50} values greater than 100 μM, except for CYP2C19 activity with PD-Ac, which had IC_{50} values of 29 μM with zero pre-incubation and 36 μM following 30 min pre-incubation. In contrast, the oligonucleotides containing phosphorothioate linkages (PT-GP and PT-Ac) had broad and varied inhibitory effects on almost all P450 and UGT enzymes evaluated. With respect to P450 enzymes, PT-GP and PT-Ac inhibited CYP1A2 most potently with direct IC_{50} values of 0.8 and 4.2 μM respectively, followed by inhibition of CYP2C8 with direct IC_{50} values of 1.1 μM and 12 μM respectively (shown in Figure 2). This inhibition was also found to be dependent on the parent phosphorothioate oligonucleotides and not the result of a low molecular weight chemical impurity or degradation product (see supplemental figure 1). Disruption of the hepatocyte plasma membrane by sonication resulted in restoration of the potent CYP1A2 and CYP2C8 inhibition for both phosphorothioate oligonucleotides (shown in supplemental figure 3). There was evidence of possible time-dependent inhibition (TDI) by PT-GP and PT-Ac of CYP2B6, CYP2C8, CYP2C19, CYP2C9, CYP2D6 and CYP3A4/5 as indicated by left-shifted IC_{50} values with pre-incubation in the absence of NADPH. The TDI for CYP2C8, which had the greatest left-shifted IC_{50} values, was found to be reversible by ultracentrifugation and re-isolation of the microsomal fraction (Figure 3). For UGT enzymes, PT-GP and PT-Ac inhibited UGT1A1 most

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potently with direct IC_{50} values of 4.5 and 5.4 μM respectively (as shown in Figure 2), followed by inhibition of UGT2B17 with direct IC_{50} values of 7.2 and 18 μM respectively. There was no evidence of TDI for UGT enzymes (evaluated at a concentration of 30 μM only) as shown in supplemental Figure 2. Overall, the rank order of direct inhibition by PT-GP was CYP1A2 ≈ CYP2C8 > UGT1A1 > UGT2B17 > CYP2B6 > UGT1A9 > CYP2C19 > UGT2B10 > UGT2B15 > UGT1A3 > UGT1A6 > CYP2D6 > CYP2C9, while the rank order of direct inhibition by PT-Ac was CYP1A2 > UGT1A1 > CYP2C8 > UGT2B17 > CYP2B6 > UGT2B10 ≈ UGT1A9 ≈ UGT2B15 ≈ UGT1A3 ≈ UGT1A6 > CYP2C19. The IC_{50} plots of the three most potently inhibited enzymes are shown in Figure 2.

Inhibition of P450 and UGT enzymes by oligonucleotides in cryopreserved human hepatocytes. To determine whether oligonucleotide inhibition of P450 and UGT enzymes was test system dependent, the four oligonucleotides (PD-GP, PT-GP, PD-Ac, PT-Ac) at 0.1 – 100 μM were evaluated as P450 and UGT inhibitors in CHH (0.5 million cells/mL) with and without 30 and 90 min pre-incubation as described in *Materials and Methods*. In this assay, overall UGT activity based on 4-methylumbelliferone glucuronidation was measured due to the lack of validated specific UGT enzyme substrates in CHH. As shown in Table 3 and Figure 4, the phosphodiester-linked oligonucleotides PD-GP and PD-Ac largely had little-to-no inhibitory effect on P450 and UGT enzymes in CHH with the exception of PD-Ac on CYP2C19 activity (direct IC_{50} of 36 μM). Similarly, the phosphorothioate-linked oligonucleotides PT-GP and PT-Ac had little-to-no direct inhibitory effect on P450 and UGT enzymes in CHH (IC_{50} values >100 μM). There was evidence of possible metabolism-dependent inhibition (MDI) for CYP2C8 and CYP3A4 by PT-GP, as IC_{50} values decreased at least by a factor of two with increasing pre-incubation time. However, in the case of CYP3A4 this inhibition was not concentration-dependent (curve not shown).

Inhibition of the uptake transporters OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2 and the efflux transporters P-gp and BCRP. Both phosphodiester and phosphorothioate variants

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of the oligonucleotides were tested as inhibitors of uptake transporters in HEK293 cell lines expressing the respective transporters and in Caco-2 or MDCK-II cells expressing efflux transporters as described in *Materials and Methods*. As shown in Table 4 and Figure 5, all four oligonucleotides had little-to-no inhibitory effect on OATP1B1, OCT1, P-gp or BCRP. For OATP1B3, only PT-GP had significant inhibition with an IC_{50} of 14 μM. PD-GP, PT-GP, PD-Ac and PT-Ac were all inhibitors of OAT1 and OCT2 with IC_{50} values spanning 12-52 μM. PT-GP and PD-Ac were also found to be inhibitors of OAT3 with IC_{50} values spanning 26-69 μM.

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DISCUSSION

To date, the publicly available data with oligonucleotide therapeutics, such as eteplirsen, mipomersen, nusinersen, volenesorsen, and defibrotide, have shown no significant pharmacokinetic drug-drug interactions (DDIs) with co-administered medicines (Adjei et al., 2003; Villalona-Calero et al., 2004; Geary et al., 2006; Yu et al., 2009; Li et al., 2014; University of Washington DDI database – http://www.druginteractioninfo.org), suggesting that, as a class, ASOs do not affect the metabolic pathways of clearance of small molecule drugs. This is consistent with the physicochemical properties of ASOs, such as their large molecular weight and polyanionic nature, as well as the assertion that these molecules are not considered prototypical substrates for drug metabolizing enzymes such as P450s. Furthermore, the proposed mechanism for oligonucleotide uptake into tissues is through endocytosis (Geary et al., 2015; Juliano and Carver, 2015), which would limit the free intracellular oligonucleotide concentration and any potential interactions with drug metabolizing enzymes located primarily in the hepatic endoplasmic reticulum. With all of this information taken together, it was unexpected to observe in vitro P450 inhibition in HLM by proprietary ASOs in the present study. Considering that phosphodiester oligonucleotides such as mRNA are endogenously present in cells as part of the gene translation process, and are not known to be inhibitors of P450 enzymes, we postulated that the phosphorothioate linkage of these ASOs was contributing to the observed in vitro P450 inhibition.

In the present study we tested this hypothesis by evaluating generic non-therapeutic oligonucleotides (partial sequences to glutathione peroxidase, GP; and beta actin, Ac) with either phosphodiester (PD) or phosphorothioate (PT) linkages as inhibitors of P450, UGT and select uptake and efflux transporters. As shown in Table 2 and Figure 2, and consistent with our internal findings with propriety ASOs, the phosphorothioate oligonucleotides evaluated in this

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study (PT-GP and PT-Ac) inhibited almost all P450 enzymes in HLM, with potent inhibition of CYP1A2 (IC_{50} values 0.8-4.2 μM) and CYP2C8 (IC_{50} values 1.1-12 μM). In addition, almost all UGT enzymes were inhibited in HLM as well, with UGT1A1 inhibited most potently (IC_{50} values 4.5-5.4 μM). In contrast, the phosphodiester oligonucleotides evaluated (PD-GP and PT-Ac) had little-to-no inhibitory effect on either P450 or UGT enzymes in HLM, with the exception of CYP2C19 for which moderate direct inhibition was observed. When all four oligonucleotides were evaluated for their ability to inhibit P450 and UGT activity in CHH, almost all of the inhibition previously observed by the phosphorothioate oligonucleotides in HLM was attenuated, indicating a clear in vitro test system-dependent inhibitory effect (see Table 3 and Figure 4). Divergent effects on enzyme inhibition with in vitro test-systems has been observed previously with some small molecule drugs (Parkinson et al., 2010). It is possible that test system-dependent inhibitory effects by phosphorothioate oligonucleotides can be attributed to the plasma membrane acting as a permeability barrier, uptake by endocytosis sequestering the oligonucleotides from P450s and UGTs, and enhanced clearance of the oligonucleotides by nucleases in CHH, all of which would reduce the inhibitory effect in CHH versus HLM. This is supported by restored potent inhibition by phosphorothioate oligonucleotides in permeabilized hepatocytes (disruption of the plasma membrane by sonication) to the same extent as HLM, but not in intact hepatocytes (see supplemental figure 3). However, given their similar structural properties, it is not clear why phosphorothioate oligonucleotides, and not phosphodiester oligonucleotides, caused potent inhibition of CYP1A2, CYP2C8 and UGT1A1 along with moderate inhibition of the other enzymes in HLM. CYP1A2 typically metabolizes aromatic and heterocyclic amines and prototypical substrates include phenacetin, tacrine, acetanilide, and caffeine (Parkinson et al., 2013). CYP2C8 substrates tend to be large acidic molecules, and substrates include rosiglitazone, repaglinide, amodiaquine, paclitaxel and several glucuronides (Ogilvie et al., 2006; Kazmi et al., 2015a; Backman et al., 2016). Lastly, UGT1A1 is involved in the glucuronidation of many substrates, including etoposide and ezetimibe (Parkinson et al.,

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2013). Chemically, oligonucleotides are composed of nucleic acid bases coupled to a ribose sugar moiety linked to other nucleotides through a 3' and 5' phosphate linkage (see Figure 1). The structural similarity between purine nucleic acid bases and CYP1A2 prototypical substrates like caffeine may explain the inhibitory finding in HLM, however this would suggest that low molecular weight degradation products of the oligonucleotides may be acting as CYP1A2 competitive inhibitors, which is not the case (see supplemental Figure 1). Furthermore, oligonucleotides are large polyanionic molecules, and share an anionic profile with CYP2C8 and UGT1A1 prototypical substrates (large acidic molecules or glucuronides). Given the anionic substrate preference for these two enzymes, perhaps CYP2C8 and UGT1A1 are more sensitive to oligonucleotide inhibition in HLM. However, both the purine and anionic interaction scenarios are not exclusive to phosphorothioate oligonucleotides, and would be expected to manifest with phosphodiester oligonucleotides as well. It is important to note that the oligonucleotides tested in this study had a low abundance of adenine, and this may have some contribution to enzyme inhibition as well. It is unclear as to the mechanism by which CYP1A2, CYP2C8 and UGT1A1 are the most potently inhibited enzymes in HLM by phosphorothioate but not phosphodiester oligonucleotides.

Time-dependent inhibition (TDI, i.e., pre-incubation without cofactor) and/or metabolism-dependent inhibition (MDI i.e., pre-incubation with cofactor) was also observed in HLM for CYP2B6, CYP2C8, CYP2C19, CYP2C9, CYP2D6 and CYP3A4/5 by the phosphorothioate but not phosphodiester oligonucleotides. In industry practices, two methodologies have been employed to determine IC_{50}-shifts, a comparison between direct and TDI IC_{50} curves or a comparison between TDI and MDI IC_{50} curves, with implications for each methodology (Parkinson et al., 2011). A shift in IC_{50} between direct and TDI IC_{50} curves implies that time alone is a factor in more potent inhibition, suggesting that in the case of the phosphorothioate oligonucleotides, a slow on-rate or impaired diffusion due to interactions between the negatively

charged microsomal phospholipid bilayer and the polyanionic oligonucleotide molecules is occurring, and not due to P450 metabolism (MDI IC_{50} shift). This was examined for CYP2C8 activity by ultracentrifugation and re-isolation of the microsomes and the TDI by the phosphorothioate oligonucleotides was shown to be reversible (see Figure 3), suggesting a slow on-rate inhibitory effect. When comparing TDI and MDI IC_{50} curves in HLM, only PT-Ac inhibition of CYP2C8 had a significant IC_{50}-shift (2.2-fold). In contrast, there was little-to-no MDI in CHH by the phosphorothioate oligonucleotides except for CYP2C8 by PT-GP, which is in contrast with the findings in HLM. An oddity is the direct inhibition of CYP2C19 by PD-Ac in both HLM and CHH, which was consistent, and it is unclear why this specific phosphodiester based oligonucleotide caused this effect; however it may suggest base sequence-specific inhibition of this enzyme.

Oligonucleotide inhibition of select uptake and efflux transporters was also investigated (see Table 4 and Figure 5), and neither phosphorothioate- nor phosphodiester-based oligonucleotides inhibited the uptake transporters OATP1B1 and OCT1 or the efflux transporters P-gp and BCRP. However, inhibition of select uptake transporters, such as OATP1B3, OAT1, OAT3, and OCT2, was observed irrespective of oligonucleotide linkage structure. Inhibition of transporters by oligonucleotides is unusual, as these biomolecules gain cellular entry through endocytosis and not through active uptake mechanisms (Geary et al., 2015; Juliano and Carver, 2015). Also, recent in vitro studies have shown no significant transporter inhibition for therapeutic phosphorothioate ASOs (Yu et al., 2016; Shemesh et al., 2017). Furthermore, a clinical interaction study with metformin, a drug transported by OCT1, OCT2 and MATE1/MATE2 (Momper et al., 2016), and a therapeutic phosphorothioate ASO (ISIS 113715) reported no clinical DDI (Geary et al., 2006). Given the lack of transporter interactions in literature reports, the inhibition of transporters by the generic oligonucleotides used in this study is through an unknown mechanism and the implications are unclear.

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Overall, our findings suggest that phosphorothioate-based oligonucleotide inhibition of P450 and UGT enzymes in HLMs is an in vitro effect without clinical relevance, and that CHHs provide a more clinically relevant inhibitory profile for use in in vitro to in vivo extrapolation (IVIVE) of DDIs. This is supported by the lack of clinical interaction reports for ASOs in the literature, which would reconcile with the lack of in vitro CHH findings by ASOs in this study. Furthermore, a recent in vitro P450 inhibition study with therapeutic phosphorothioate-based ASOs and CHH has shown a lack of in vitro inhibition and consequently no predicted in vivo DDI (Shemesh et al., 2017), consistent with the results of this study. As a result, we recommend that, for phosphorothioate-based ASOs as a molecule class, P450 and UGT inhibition studies be performed in CHH and not in HLM under validated conditions, which include substrate concentrations at approximate K_m with short incubation times to prevent extensive substrate depletion.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Kazmi, Parkinson, Buckley, Ogilvie.

Conducted Experiments: Yerino, McCoy.

Performed data analysis: Kazmi, Yerino, Buckley.

Wrote or contributed to the writing of the manuscript: Kazmi, Parkinson, Buckley, Ogilvie.

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FOOTNOTES:

Portions of this work were previously presented at the following meetings: Kazmi F, Yerino P, Bixler E, McCoy C, and Buckley DB (2014) In vitro system-dependent inhibition of cytochrome P450 enzymes (CYP), UGP-glucuronosyltransferases (UGT) and transporters by oligonucleotides. *Nineteenth North American Regional ISSX Meeting*; 2014 Oct 19-23; San Francisco, CA. International Society for the Study of Xenobiotics, Washington DC; Buckley D, Kazmi F, Yerino P, Ogilvie B, and Parkinson A (2009) Inhibition of cytochrome P450 (CYP) enzymes, CYP1A2 and CYP2C8, by oligonucleotides in human liver microsomes (HLM): A system-dependent outcome. *Sixteenth North American Regional ISSX Meeting*; 2009 Oct 18-22; Baltimore, MD. International Society for the Study of Xenobiotics.

FIGURE LEGENDS

Fig. 1: General structures of oligonucleotides with phosphodiester or phosphorothioate linkages

Fig. 2: IC_{50} plots of CYP1A2, CYP2C8 and UGT1A1 inhibition by phosphodiester (PD) or phosphorothioate (PT) linked oligonucleotides in human liver microsomes (HLM). As described in *Materials and Methods*, the inhibition of CYP1A2 (phenacetin), CYP2C8 (amodiaquine) and UGT1A1 (estradiol) activity was assessed at 0.1-100 μM oligonucleotide in HLM at ≤ 0.1 mg/mL, with or without a pre-incubation in the absence of NADPH cofactor (30 min) for the evaluation of direct and time-dependent inhibition (TDI) respectively, or in the presence of NADPH cofactor for the evaluation of metabolism-dependent inhibition (MDI).

Fig. 3: Assessment of the reversibility of CYP2C8 time-dependent inhibition (TDI) by phosphorothioate (PT) linked oligonucleotides in human liver microsomes (HLM). PT oligonucleotides were incubated at 10 μM for 30 min in HLM (0.0125 mg/mL), followed by ultracentrifugation (100,000 x g) and re-isolation of the microsomal fraction as described in *Materials and Methods*. Residual CYP2C8 (amodiaquine) activity was assessed following protein normalization.

Fig. 4: IC_{50} plots of CYP1A2, CYP2C8 and UGT inhibition by phosphodiester (PD) or phosphorothioate (PT) linked oligonucleotides in cryopreserved human hepatocytes (CHH). As described in *Materials and Methods*, the inhibition of CYP1A2 (phenacetin), CYP2C8 (amodiaquine) and overall UGT (4-methylumbelliferone) activity (estradiol) was assessed at 0.1-100 μM oligonucleotide in CHH at 0.5 million cells/mL, with or without a pre-incubation (30 and 90 min) for the evaluation of direct and metabolism-dependent inhibition (MDI).

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Fig. 5: IC_{50} plots of OATP1B3, OAT1, OAT3, and OCT2 inhibition by phosphodiester (PD) or phosphorothioate (PT) linked oligonucleotides. As described in *Materials and Methods*, the inhibition of OATP1B3 (estradiol 17B-glucuronide), OAT1 (p-Aminohippuric acid), OAT3 (estrone 3-sulfate) and OCT2 (metformin) activity was assessed at 0.1-100 μM oligonucleotide in transporter overexpressing HEK293 cells.

Table 1: Experimental conditions for measurement of P450 and UGT activity in human liver microsomes (HLM) and cryopreserved human hepatocytes (CHH)

Enzyme	Marker activity	Substrate concentration (μM)	HLM (mg/mL)	CHH (million cells/mL)	Incubation time in HLM (min)	Incubation time in CHH (min)	Mass transition monitored (m/z)	Ionization mode	Internal Standard
CYP1A2	Phenacetin *O*-deethylation	40	0.1	0.5	5	10	150.0 / 107.0	Negative	d_4-Acetaminophen
CYP2B6	Bupropion hydroxylation	50	0.1	0.5	5	10	256.1 / 238.0	Positive	d_6-Hydroxybupropion
CYP2C8	Amodiaquine *N*-dealkylation	1.5 (7 for CHH)	0.0125	0.5	5	10	328.2 / 283.1	Positive	d_5-*N*-Desethylamodiaquine
CYP2C9	Diclofenac 4′-hydroxylation	6	0.1	0.5	5	10	310.0 / 266.0	Negative	d_4-4′-Hydroxydiclofenac
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	40	0.1	0.5	5	10	233.0 / 190.0	Negative	d_3-4′-Hydroxymephenytoin
CYP2D6	Dextromethorphan O-demethylation	7.5	0.1	0.5	5	10	258.0 / 157.1	Positive	d_3-Dextrorphan
CYP3A4/5	Midazolam 1′-hydroxylation	4	0.1	0.5	5	10	342.0 / 324.0	Positive	d_4-1′-Hydroxymidazolam
UGT1A1	Estradiol 3-*O*-glucuronidation	9	0.1	NA	5	NA	4471 / 271.2	Negative	d_5-Estradiol 3-*O*-glucuronide
UGT1A3	Chenodeoxycholic acid 24-*O*-glucuronidation	20	0.1	NA	10	NA	567.2 / 391.3	Negative	d_5-Chenodeoxycholic acid 24-*O*-glucuronide
UGT1A4	Trifluoperazine glucuronidation	12	0.1	NA	5	NA	584.0 / 408.0	Positive	Prochlorperazine glucuronide
UGT1A6	1-Naphthol glucuronidation	1	0.0125	NA	5	NA	319.1 / 143.1	Negative	d_7-Naphthol glucuronide
UGT1A9	Propofol glucuronidation	20	0.1	NA	5	NA	353.2 / 177.1	Negative	d_{17}-Propofol glucuronide
UGT2B7	Morphine 3-*O*-glucuronidation	400	0.1	NA	5	NA	462.0 / 286.0	Positive	d_3-Morphine 3-*O*-glucuronide
UGT2B10	Levomedetomidine glucuronidation	7	0.1	NA	10	NA	377.0 / 201.0	Positive	d_4-1′-Hydroxymidazolam
UGT2B15	*S*-Oxazepam glucuronidation	50	0.1	NA	10	NA	463.0 / 286.4	Positive	d_5-*S*-Oxazepam glucuronide
UGT2B17	Testosterone 17-*O*-glucuronidation	5	0.1	NA	10	NA	465.1 / 289.0	Positive	d_5-Testosterone 17-*O*-glucuronide
UGT	4-Methylumbelliferone glucuronidation	70	0.1	0.5	NA	10	352.9 / 177.1	Positive	d_5-7-Hydroxycoumarin glucuronide

NA, not applicable

Table 2: IC_{50} values for P450 and UGT inhibition with phosphodiester (PD) or phosphorothioate (PT) oligonucleotides in human liver microsomes (HLM)

Enzyme	Substrate	IC_{50} (μM)											
		PD-GP			PT-GP			PD-Ac			PT-Ac		
		Direct	TDI	MDI	Direct	TDI	MDI	Direct	TDI	MDI	Direct	TDI	MDI
CYP1A2	Phenacetin	>100	>100	>100	0.8	0.8	1.8	>100	>100	>100	4.2	3.4	7.3
CYP2B6	Bupropion	>100	>100	>100	15	15	13	>100	>100	>100	39	20	22
CYP2C8	Amodiaquine	>100	>100	>100	1.1	0.6	0.6	>100	>100	>100	12	8.3	3.7
CYP2C9	Diclofenac	>100	>100	>100	97	36	47	>100	>100	>100	>100	53	57
CYP2C19	S-Mephenytoin	>100	>100	>100	21	8.7	19	29	36	>100	80	39	98
CYP2D6	Dextromethorphan	>100	>100	>100	81	41	>100	>100	>100	>100	>100	52	>100
CYP3A4/5	Midazolam	>100	>100	>100	>100	38	28	>100	>100	>100	>100	66	63
UGT1A1	Estradiol	>100	ND[1]	ND[1]	4.5	ND[1]	ND[1]	>100	ND[1]	ND[1]	5.4	ND[1]	ND[1]
UGT1A3	Chenodeoxycholic acid	>100	ND[1]	ND[1]	51	ND[1]	ND[1]	>100	ND[1]	ND[1]	48	ND[1]	ND[1]
UGT1A4	Trifluoperazine	>100	ND[1]	ND[1]	>100	ND[1]	ND[1]	>100	ND[1]	ND[1]	>100	ND[1]	ND[1]
UGT1A6	1-Naphthol	>100	ND[1]	ND[1]	52	ND[1]	ND[1]	>100	ND[1]	ND[1]	55	ND[1]	ND[1]
UGT1A9	Propofol	>100	ND[1]	ND[1]	19	ND[1]	ND[1]	>100	ND[1]	ND[1]	42	ND[1]	ND[1]
UGT2B7	Morphine	>100	ND[1]	ND[1]	>100	ND[1]	ND[1]	>100	ND[1]	ND[1]	>100	ND[1]	ND[1]
UGT2B10	Levomedetomidine	>100	ND[1]	ND[1]	26	ND[1]	ND[1]	>100	ND[1]	ND[1]	41	ND[1]	ND[1]
UGT2B15	S-Oxazepam	>100	ND[1]	ND[1]	38	ND[1]	ND[1]	>100	ND[1]	ND[1]	45	ND[1]	ND[1]
UGT2B17	Testosterone	>100	ND[1]	ND[1]	7.2	ND[1]	ND[1]	>100	ND[1]	ND[1]	18	ND[1]	ND[1]

PD-GP, phosphodiester glutathione peroxidase oligonucleotide; PT-GP, phosphorothioate glutathione peroxidase oligonucleotide; PT-Ac, phosphodiester beta actin oligonucleotide; PT-Ac, phosphorothioate beta actin oligonucleotide; ND, not done.
[1] Performed at 30 μM oligonucleotide as shown in Supplemental Figure 2

Table 3: IC_{50} values for P450 and UGT inhibition with phosphodiester (PD) or phosphorothioate (PT) oligonucleotides in cryopreserved human hepatocytes (CHH)

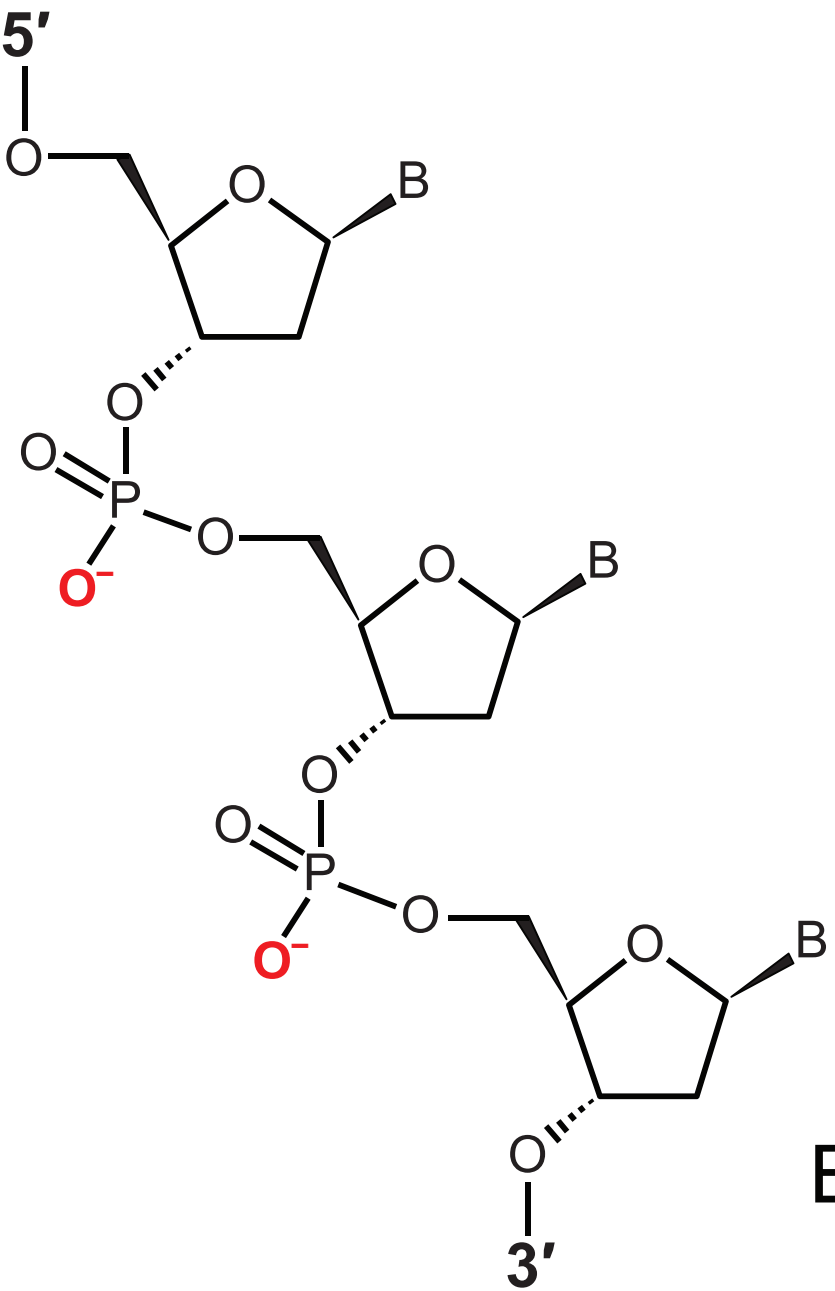
Enzyme	Substrate	IC_{50} (μM)											
		PD-GP			PT-GP			PD-Ac			PT-Ac		
		Direct	MDI 30 min	MDI 90 min	Direct	MDI 30 min	MDI 90 min	Direct	MDI 30 min	MDI 90 min	Direct	MDI 30 min	MDI 90 min
CYP1A2	Phenacetin	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
CYP2B6	Bupropion	>100	>100	>100	>100	>100	>100	>100	>100	77	>100	>100	>100
CYP2C8	Amodiaquine	>100	>100	>100	>100	35	44	>100	>100	>100	>100	>100	>100
CYP2C9	Diclofenac	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
CYP2C19	S-Mephenytoin	>100	>100	>100	>100	>100	>100	36	>100	>100	>100	>100	>100
CYP2D6	Dextromethorphan	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
CYP3A4/5	Midazolam	>100	>100	>100	>100	>100	49	>100	>100	>100	>100	>100	>100
UGT	4-Methylumbelliferone	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

PD-GP, phosphodiester glutathione peroxidase oligonucleotide; PT-GP, phosphorothioate glutathione peroxidase oligonucleotide; PT-Ac, phosphodiester beta actin oligonucleotide; PT-Ac, phosphorothioate beta actin oligonucleotide.

Table 4: IC_{50} values for uptake and efflux transporter inhibition with phosphodiester (PD) or phosphorothioate (PT) oligonucleotides

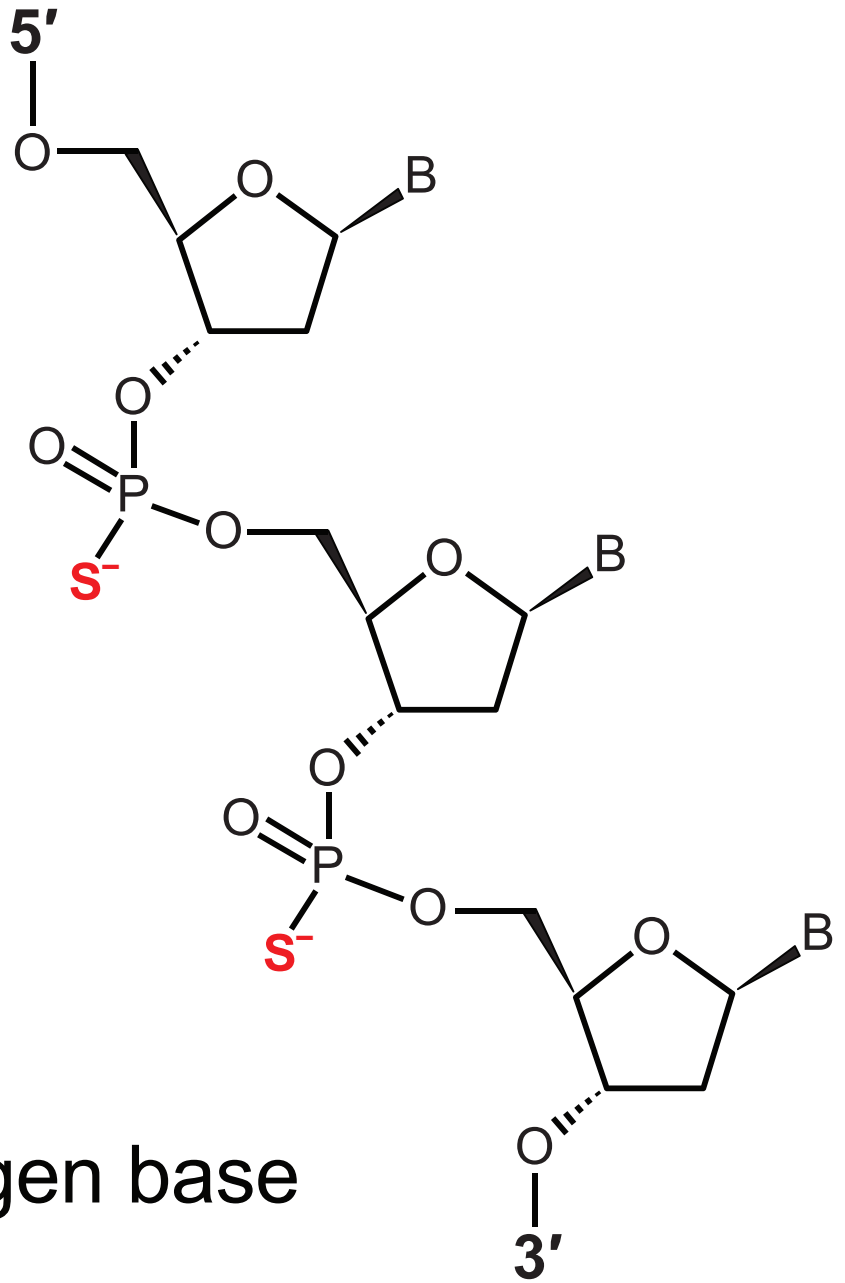
Transporter	Substrate	IC_{50} (μM)			
		PD-GP	PT-GP	PD-Ac	PT-Ac
OATP1B1	Estradiol 17-β-glucuronide	>100	90	>100	>100
OATP1B3	Estradiol 17-β-glucuronide	>100	14	>100	92
OAT1	*p*-Aminohippuric acid	46	12	52	16
OAT3	Estrone 3-sulfate	>100	26	69	>100
OCT1	Tetraethylammonium bromide	>100	>100	>100	>100
OCT2	Metformin	17	14	27	29
P-gp	Digoxin	>100	>100	>100	>100
BCRP	Prazosin	>100	>100	>100	>100

PD-GP, phosphodiester glutathione peroxidase oligonucleotide; PT-GP, phosphorothioate glutathione peroxidase oligonucleotide; PT-Ac, phosphodiester beta actin oligonucleotide; PT-Ac, phosphorothioate beta actin oligonucleotide.



Phosphodiester oligo

B = nitrogen base



Phosphorothioate oligo

Figure 1

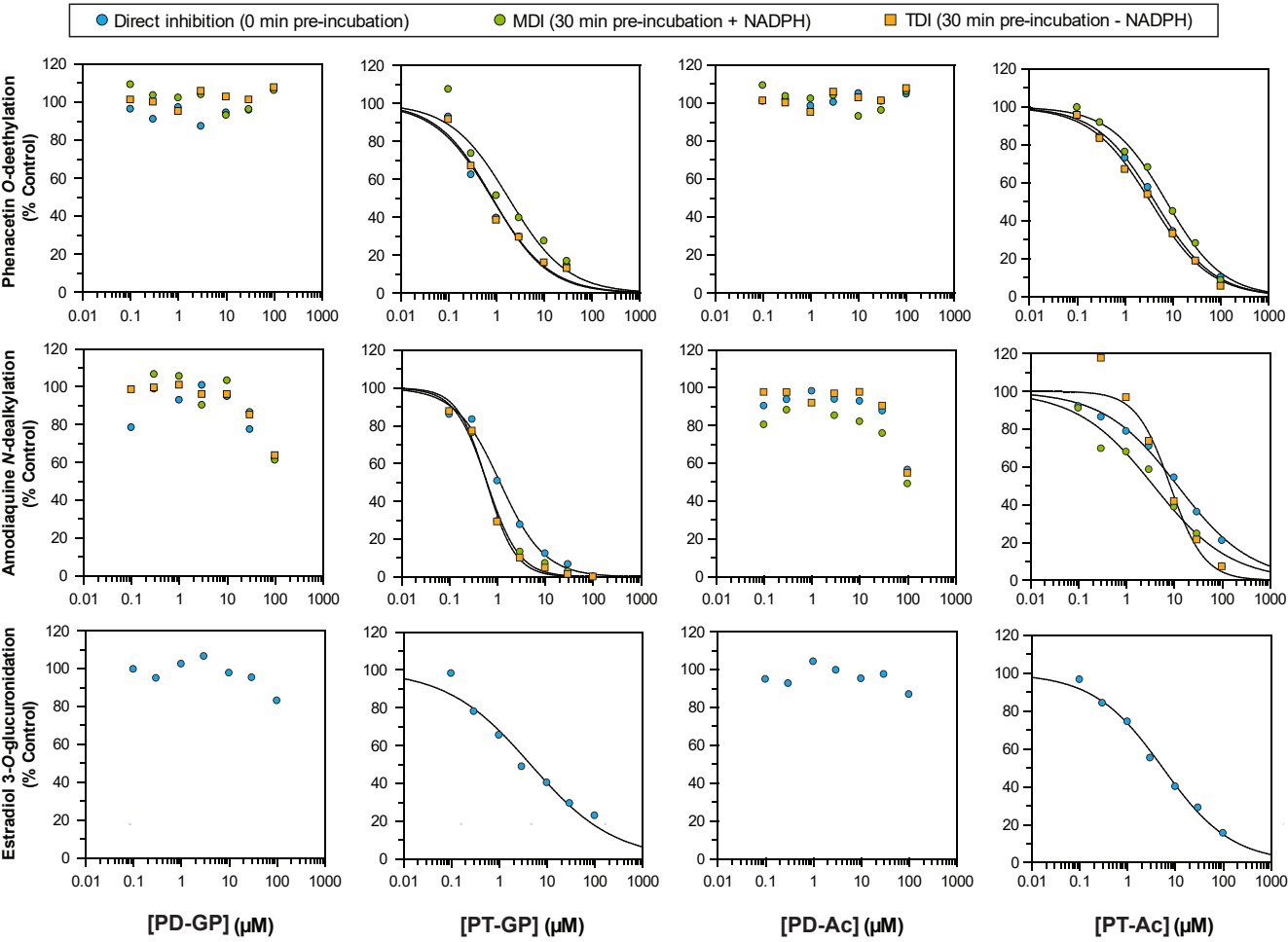

Direct inhibition (0 min pre-incubation)
MDI (30 min pre-incubation + NADPH)
TDI (30 min pre-incubation - NADPH)
Phenacetin O-deethylation (% Control)
Amodiaquine N-dealkylation (% Control)
Estradiol 3-O-glucuronidation (% Control)
[PD-GP] (µM)
[PT-GP] (µM)
[PD-Ac] (µM)
[PT-Ac] (µM)

Figure 2

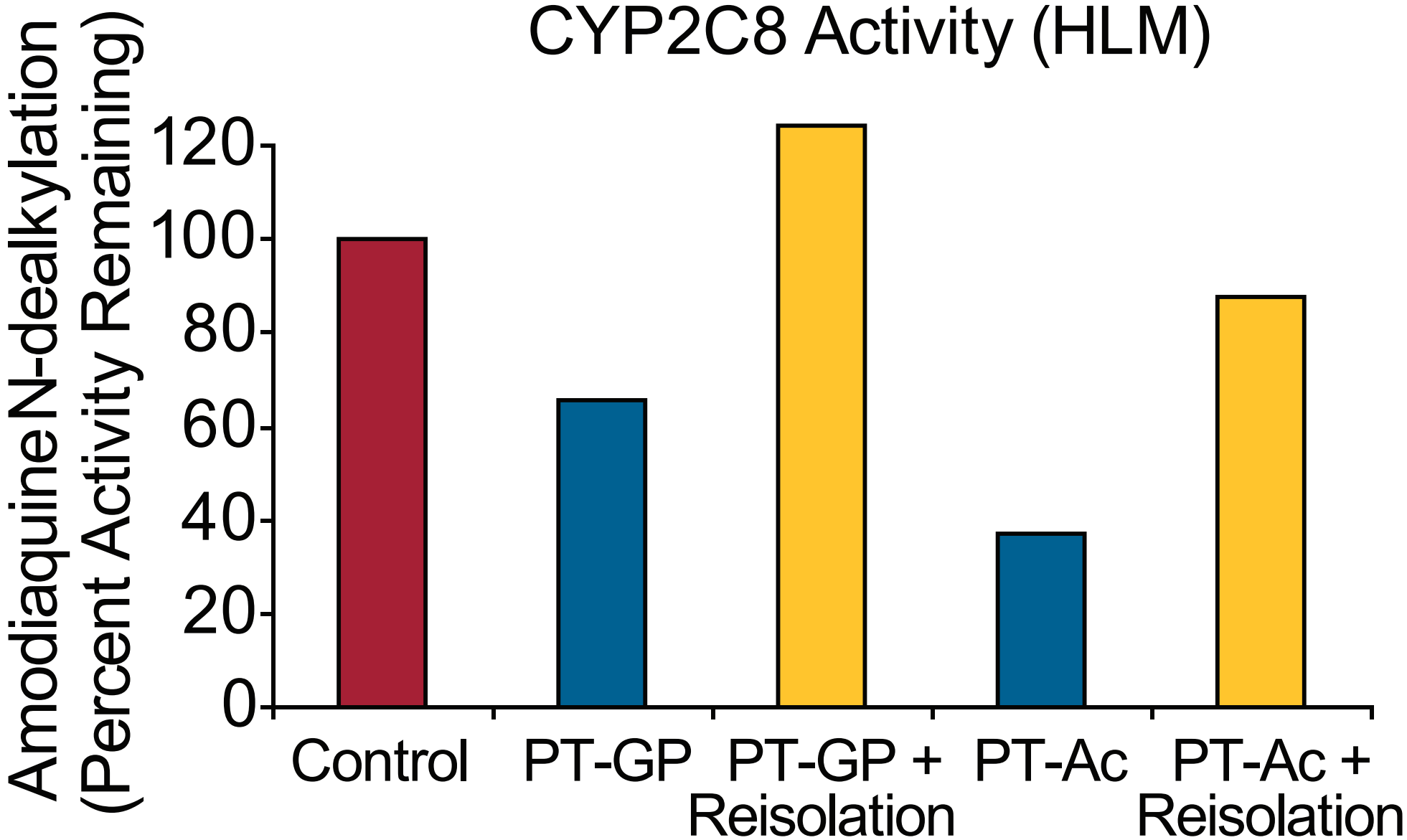

CYP2C8 Activity (HLM)
Amodiaquine N-dealkylation
(Percent Activity Remaining)
120
100
80
60
40
20
0
Control
PT-GP
PT-GP + Reisolation
PT-Ac
PT-Ac + Reisolation

Figure 3

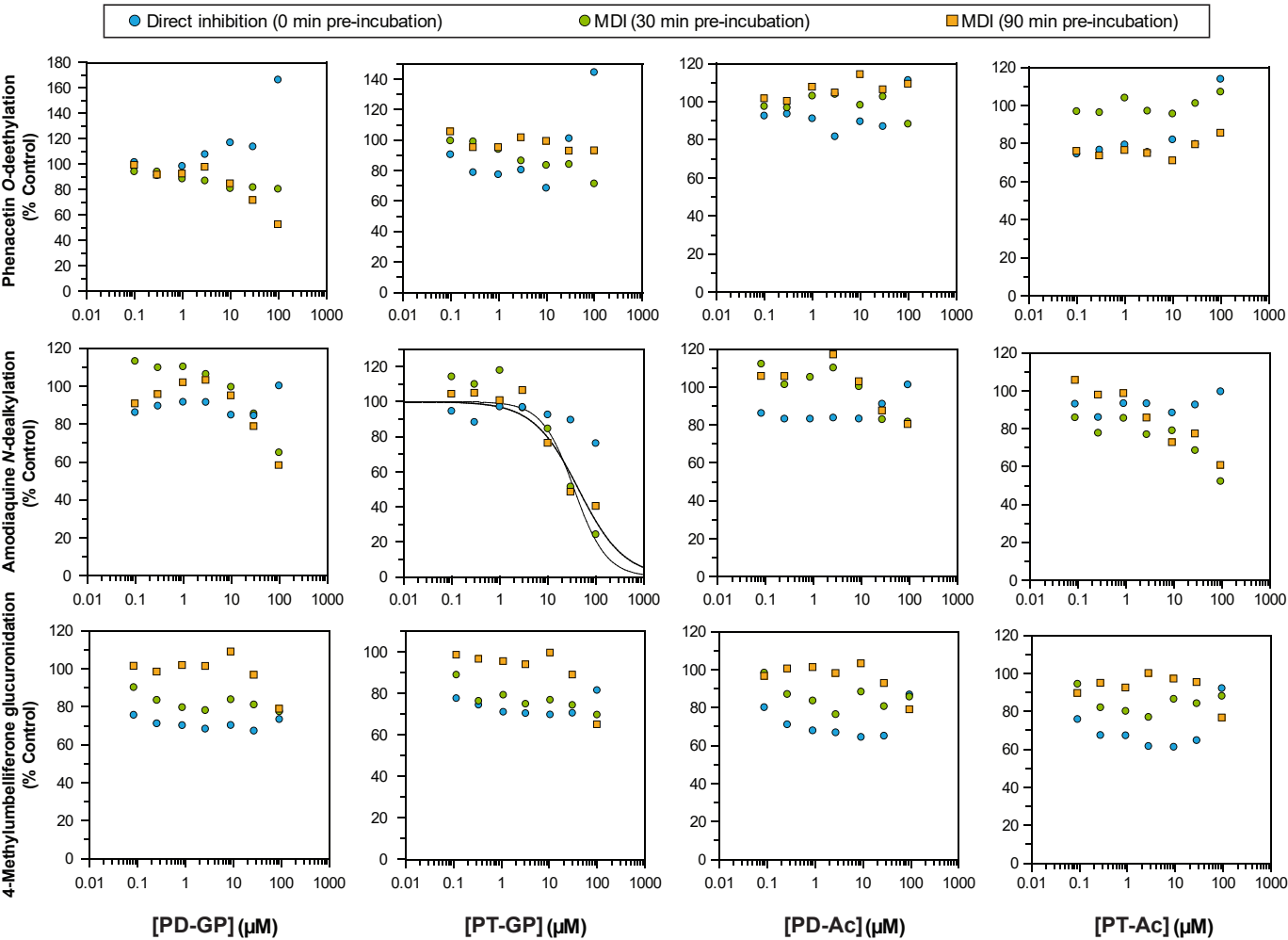

Direct inhibition (0 min pre-incubation)
MDI (30 min pre-incubation)
MDI (90 min pre-incubation)
Phenacetin O-deethylation (% Control)
Amodiaquine N-dealkylation (% Control)
4-Methylumbelliferone glucuronidation (% Control)
[PD-GP] (μM)
[PT-GP] (μM)
[PD-Ac] (μM)
[PT-Ac] (μM)

Figure 4

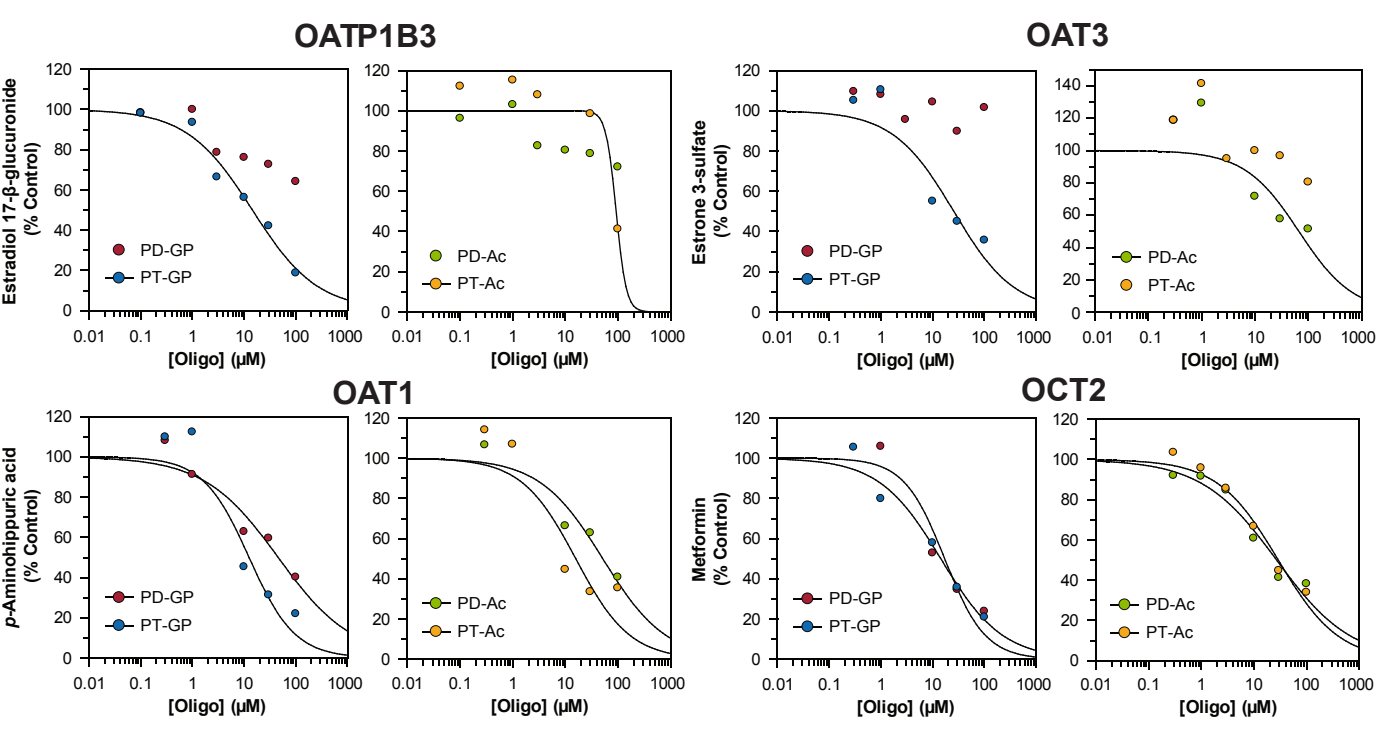

OATP1B3
OAT3
OAT1
OCT2
Estradiol 17-β-glucuronide (% Control)
Estrone 3-sulfate (% Control)
p-Aminohippuric acid (% Control)
Metformin (% Control)
PD-GP
PT-GP
PD-Ac
PT-Ac
[Oligo] (μM)
0.01
0.1
1
10
100
1000
0
20
40
60
80
100
120
140

Figure 5

Supplemental Materials

An assessment of the *in vitro* inhibition of cytochrome P450 enzymes (CYP), UDP-glucuronsyltransferases (UGT) and transporters by phosphodiester- or phosphorothioate-linked oligonucleotides

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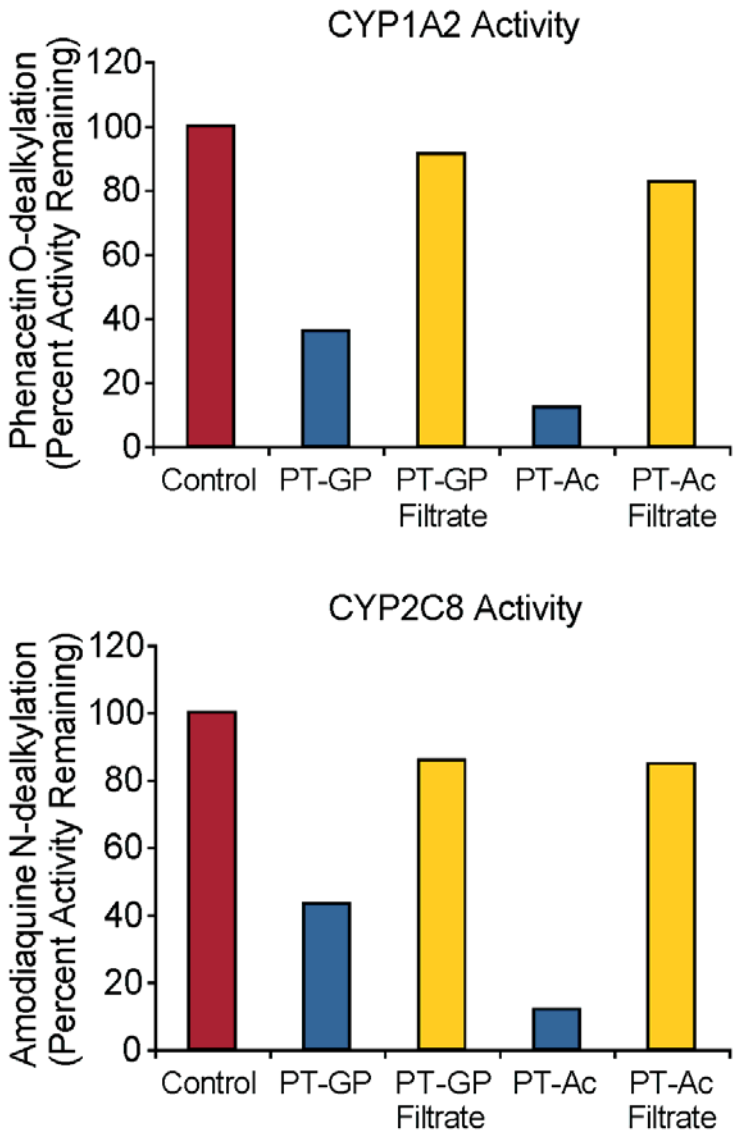
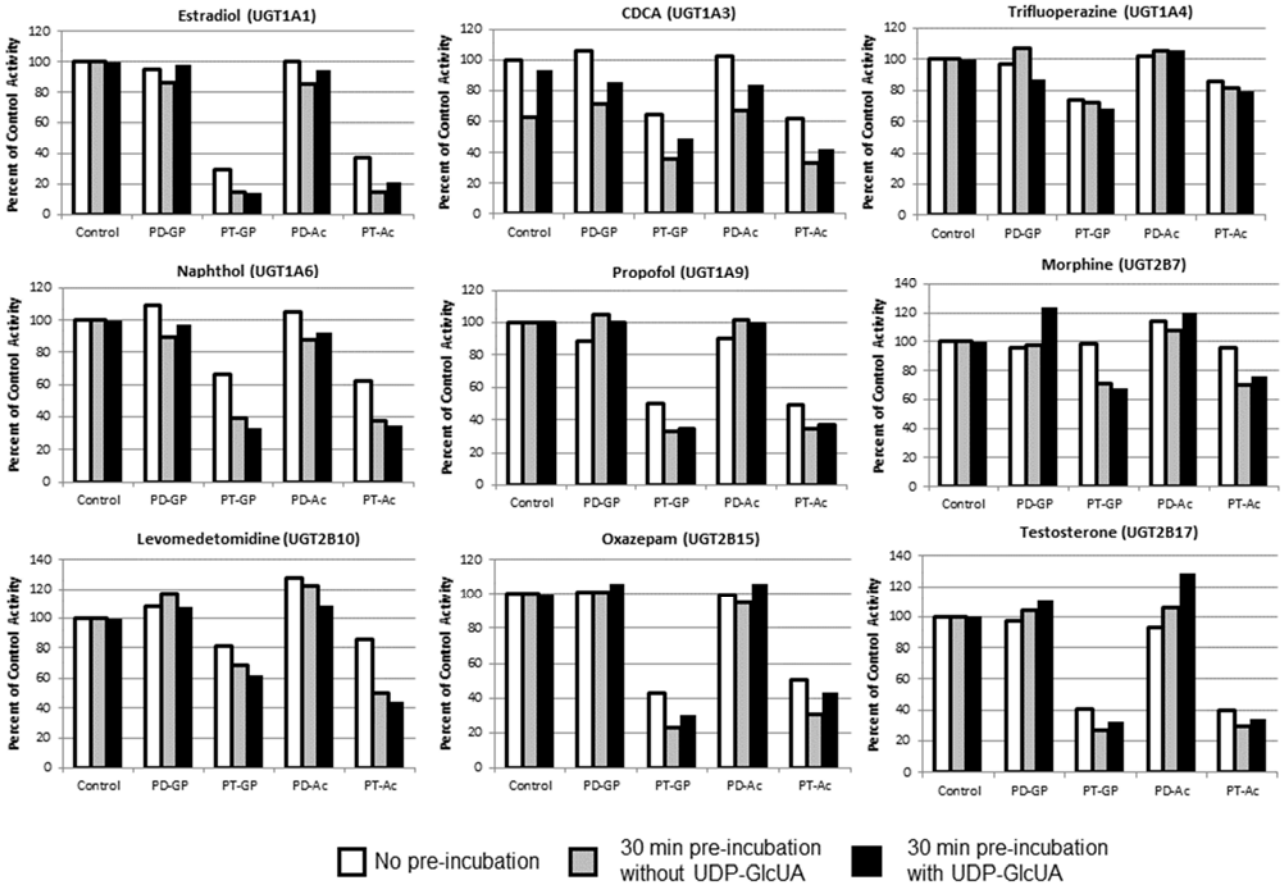
Janssen Research & Development, LLC, Spring House, PA, USA (F.K.)
XPD Consulting, Shawnee, KS, USA (A.P.)
Roivant Sciences, Durham, NC, USA (D.B.B.)

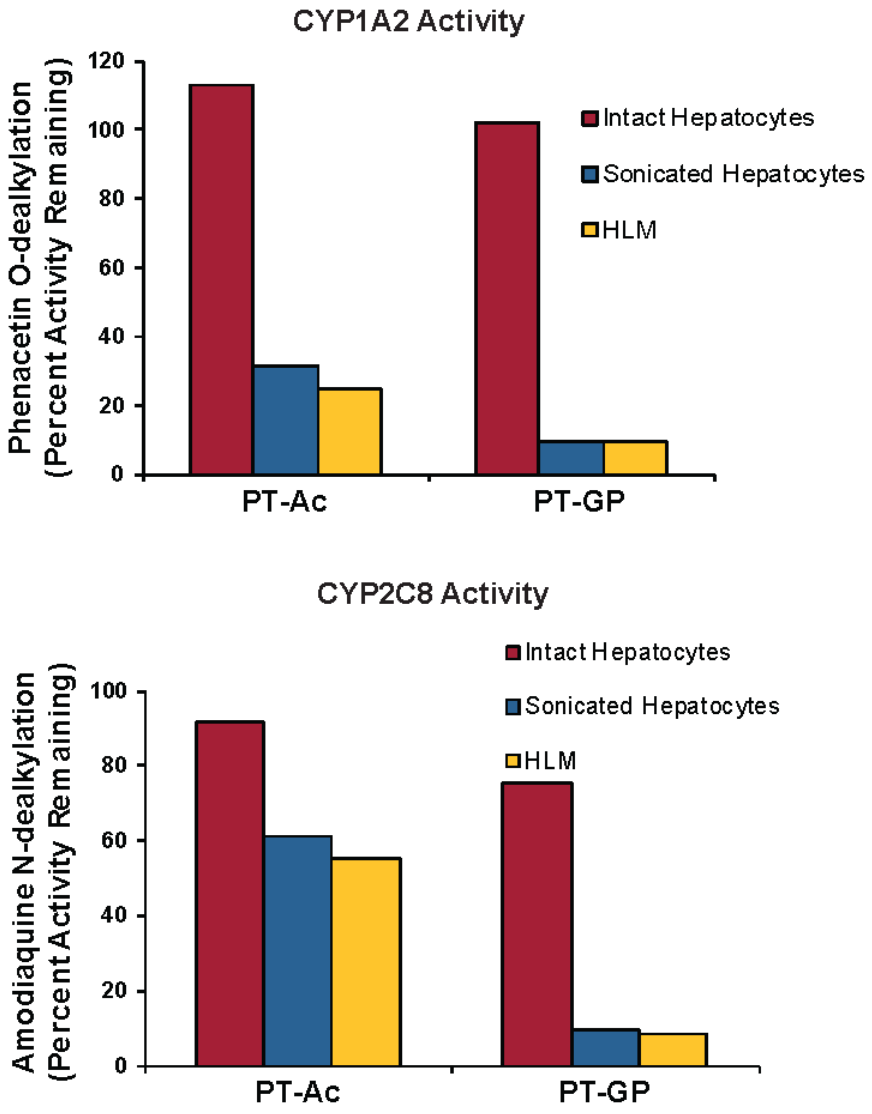
Drug Metabolism and Disposition



Supplemental Figure 1: Assessment of low molecular weight (3 kDa) filtration on the inhibitory effect of phosphorothioate (PT) oligonucleotides in human liver microsomes (HLM).As described in the *Materials and Methods*, the inhibition of CYP1A2 (phenacetin) and CYP2C8 (amodiaquine) by phosphorothioate (PT) based glutathione perioxidase (GP) or beta-actin (Ac) oligonucleotides was assessed at 100 μM in HLM (≤ 0.1 mg/mL) for 5 min with and without prior filtration of the PT oligonucleotides through a 3 kDa filter to remove low molecular weight impurities and degradation products. Reactions were terminated after 5 min followed by LC-MS/MS analysis.



Supplemental Figure 2: The evaluation of time dependent inhibition (TDI) of UDP-glucuronsyltransferases (UGT) by phosphodiester (PD) or phosphorothioate (PT) linked oligonucleotides in human liver microsomes (HLM). As described in *Materials and Methods*, the oligonucleotides (at 30 μM) were incubated with HLM (≤ 0.1 mg/mL) with and without at 30 min pre-incubation in the presence or absence of UDP-GlcUA. Reactions were terminated after 5 or 10 min followed by LC-MS/MS analysis.



Supplemental Figure 3: Assessment of CYP1A2 and CYP2C8 inhibition by phosphorothioate (PT) oligonucleotides in intact cryopreserved human hepatocytes (CHH), sonicated CHH, and human liver microsomes (HLM). Briefly, the PT oligonucleotides (at 10 μM) were incubated with 0.5 million cells/mL CHHs that were either intact (red bars) or disrupted with probe sonication (blue bars), and ≤ 0.1 mg/mL HLM (yellow bars) as described in *Materials and Methods*. Substrate incubations for CYP1A2 (phenacetin) and CYP2C8 (amodiaquine) were conducted as described in Table 1, followed by LC-MS/MS analysis.