In Vitro System-Dependent Inhibition of Cytochrome P450 Enzymes (CYP), UDP-Glucuronosyl Transferases (UGT) and Transporters by Oligonucleotides

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Introduction

Oligonucleotides represent a growing class of biotherapeutics currently being developed for a variety of indications. They are often developed as antisense molecules (of approximately 20 nucleotides in length) designed to hybridize to a target mRNA molecule, preventing its translation to a protein. In many cases, these large polyanionic molecules are developed with phosphorothioate backbones to improve their biological stability. Phosphorothioate oligonucleotides are biopharmaceutics classification system (BCS) class III compounds, such that they have high aqueous solubility but low intestinal permeability, and are highly bound to plasma proteins (Geary, 2009; Yu *et al.*, 2013). Despite the increased pharmaceutical development of phosphorothioate oligonucleotides, there are limited drug-drug interaction (DDI) data available on this class of biotherapeutics.

We previously examined the cytochrome P450 (CYP) inhibition potential of oligonucleotides and reported that the phosphorothioate, but not phosphodiester, backboned molecules caused potent inhibition of CYP1A2 and CYP2C8 in human liver microsomes (HLM) but not in cryopreserved human hepatocytes (CHH) (Buckley *et al.*, 2009). In this study we expanded upon our previous work to include direct, time- (TDI) and metabolism-dependent inhibition (MDI) of 7 CYP and 8 UGT enzymes in pooled HLM and pooled CHH. Additionally, inhibition of the transporters OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, Pgp and BCRP was also examined.

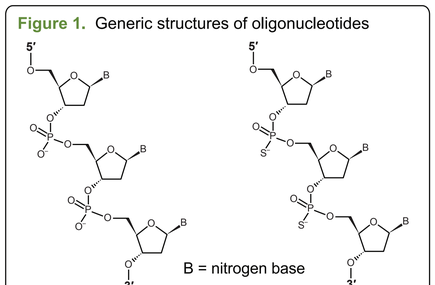
Materials & Methods

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 backbone (generic structures shown in Figure 1) were purchased from Integrated DNA Technologies (Coralville, IA).

Legend	Oligonucleotide
Oligo #1	Glutathione peroxidase phosphodiester backbone
Oligo #2	Glutathione peroxidase phosphorothioate backbone
Oligo #3	Beta actin phosphodiester backbone
Oligo #4	Beta actin phosphorothioate backbone

Figure 1. Generic structures of oligonucleotides



Chemicals

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 all purchased from Sigma-Aldrich (St. Louis, MO). Levomedetomidine was a gift from Orion Corporation (Espoo, Finland). [3H]-Estradiol glucuronide and [3H]-Estrone sulfate were purchased from Perkin Elmer (Waltham, MA). [14C]-Metformin was purchased from Moravek Biochemicals (Brea, CA). [14C]-Tetraethylammonium bromide and [3H]-*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 in-house. 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).

Test system

Pooled human liver microsomes (HLM, n = 200, mixed gender) and pooled cryopreserved human hepatocytes (CHH, n = 100, mixed gender) were prepared from non-transplantable livers and characterized at XenoTech, LLC (Lenexa, 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) will be used. HEK293 cells were supplied from 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* CYP and UGT inhibition**

The effects of oligonucleotides on CYP enzymes in HLM were evaluated in IC_{50} experiments with and without a preincubation step (in the presence and absence of NADPH) as described previously (Parkinson *et al.*, 2011). For CHH, incubations were conducted in 100 µl mixtures at 0.5 million cells/mL in KHB. Reactions were initiated with the addition of CHH to the oligos 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 CYP marker substrate (or the broad specific UGT substrate 4-methylumbelliferone; 4-MU) at a concentration approximately equal to its K_m (see Table 1). 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.

For UGT inhibition assays, oligos were incubated 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), UDPGA (10 mM), and a UGT marker substrate at a concentration approximately equal to its K_m, at the final concentrations indicated in Table 1. Reactions were initiated by the addition of UDPGA 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 oligo (30 µM) with and without a 30 min preincubation step (in the presence and absence of UDPGA) with the methodology described above. IC_{50} values were determined with GraFit (version 7.0.2; Erithricus Software Ltd., Surrey, UK).

***In vitro* uptake and efflux transporter inhibition**

To evaluate inhibition of uptake transporters, HEK293 cells over-expressing various 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 oligos or solvent control for 15 min followed by 2 min incubation with a combination of probe substrate (a mixture of cold and radiolabeled compound) and oligo. 50 nM estradiol 17-β-glucuronide substrate was used for OATP1B1 and OATP1B3 assays (with approximate equimolar radio-labeled 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 tetraethyl-ammonium 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) 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 in the presence of oligonucleotides. Oligos 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. IC_{50} values were determined with GraFit (version 7.0.2; Erithricus Software Ltd., Surrey, UK).

Results

As shown in Table 2, Figure 2 and Table 3, the results indicated that oligos #1 and #3 with the phosphodiester backbones had little to no inhibitory effect on all CYP and UGT enzymes in HLM and CHH with the exception of oligo #3 in HLM for CYP2C19. Conversely, the oligos with the phosphorothioate backbone caused direct inhibition of CYP and UGT enzymes. The rank order of direct inhibition in HLM for oligo #2 was CYP1A2 > CYP2C8 > UGT1A1 > UGT2B17 > CYP2B6 > UGT1A9 > CYP2C19 > UGT2B10 > UGT2B15 > UGT1A3 > UGT1A6 > CYP2D6 > CYP2C9. Similar results were observed for oligo #4: CYP1A2 > UGT1A1 > CYP2C8 > UGT2B17 > CYP2B6 > UGT2B10 > UGT1A9 > UGT2B15 > UGT1A3 > UGT1A6 > CYP2C19.

Results (cont.)

There was evidence of time-dependent inhibition (TDI) of CYP enzymes in HLM in the absence of NADPH-with oligo #2 and #4 for CYP2B6, CYP2C8, CYP2C19, CYP2C9, CYP2D6 and CYP3A4/5. Little to no TDI was observed for UGT enzymes in HLM (data not shown). In contrast to HLM, as shown in Table 3 and Figure 3, we observed little to no direct inhibition by any oligo in CHH (with the exception of oligo #3 with CYP2C19 and TDI by oligo #2 with CYP2C8), demonstrating system-dependent outcomes. There was moderate inhibition of CYP3A4/5 in CHH by oligo #2 after a 90 min pre-incubation; however, this inhibition was not concentration dependent and not considered significant despite yielding an IC_{50} value (49 µM).

The oligos were also tested as inhibitors of uptake and efflux transporters and the results are shown in Table 4 and Figure 4. In summary, oligo #1 was found to inhibit OAT1 and OCT2; oligo #2 was found to inhibit OATP1B1, OATP1B3, OAT1, OAT3 and OCT2; oligo #3 was found to inhibit OAT1, OAT3 and OCT2; and oligo #4 was found to inhibit OATP1B3, OAT1 and OCT2. While phosphorothioate oligos overall inhibited more uptake transporters, phosphodiester oligos were inhibitory towards OAT1, OAT3 and OCT2. None of the oligos significantly inhibited the efflux transporters Pgp and BCRP.

Table 1. Experimental conditions for measurement of CYP and UGT activity for HLM and CHH studies

Enzyme	CYP or UGT 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)	Internal Standard
CYP1A2	Phenacetin *O*-deethylation	40	0.1	0.5	5	10	152 / 110	d_4-Acetaminophen
CYP2B6	Bupropion hydroxylation	50					256 / 238	d_6-Hydroxybupropion
CYP2C8	Amodiaquine *N*-dealkylation	1.5 (7 for CHH)	0.0125				328 / 283	d_5-*N*-Desethylamodiaquine
CYP2C9	Diclofenac 4′-hydroxylation	6	0.1				310 / 266	d_4-4′-Hydroxydiclofenac
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	40					233 / 190	d_3-4′-Hydroxymephenytoin
CYP2D6	Dextromethorphan *O*-demethylation	7.5					258 / 157	d_3-Dextrorphan
CYP3A4/5	Midazolam 1′-hydroxylation	4					342 / 324	d_4-1′-Hydroxymidazolam
UGT1A1	Estradiol 3-*O*-glucuronidation	9	0.1	NA	5	NA	447 / 271	d_5-Estradiol 3-*O*-glucuronide
UGT1A3	Chenodeoxycholic acid 24-*O*-glucuronidation	20			10		567 / 391	d_5-Chenodeoxycholic acid 24-*O*-glucuronide
UGT1A4	Trifluoperazine glucuronidation	12					584 / 408	Prochlorperazine glucuronide
UGT1A6	1-Naphthol glucuronidation	1	0.0125		5		319 / 143	d_7-Naphthol glucuronide
UGT1A9	Propofol glucuronidation	20	0.1				353 / 177	d_{17}-Propofol glucuronide
UGT2B7	Morphine 3-*O*-glucuronidation	400					462 / 286	d_3-Morphine 3-*O*-glucuronide
UGT2B10	Levomedetomidine glucuronidation	7			10		377 / 201	d_4-1′-Hydroxymidazolam
UGT2B15	*S*-Oxazepam glucuronidation	50					463 / 286	d_5-*S*-Oxazepam glucuronide
UGT2B17	Testosterone 17-*O*-glucuronidation	5					465 / 289	d_5-Testosterone 17-*O*-glucuronide
UGT	4-Methylumbelliferone glucuronidation	70		0.5	NA	10	353 / 177	d_5-7-Hydroxycoumarin glucuronide

NA: Not applicable HLM: Human liver microsomes CHH: Cryopreserved human hepatocytes

Table 2. IC_{50} values for CYP and UGT inhibition with oligonucleotides in HLM

Enzyme	Substrate	IC_{50} (µM)											
		Oligo #1			Oligo #2			Oligo #3			Oligo #4		
		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				15	15	13				39	20	22
CYP2C8	Amodiaquine				1.1	0.6	0.6				12	8.3	3.8
CYP2C9	Diclofenac				97	36	47				>100	53	57
CYP2C19	*S*-Mephenytoin				21	8.7	19	29	36		80	39	98
CYP2D6	Dextromethorphan				81	41	>100	>100	>100	>100	>100	52	>100
CYP3A4/5	Midazolam				>100	38	28					66	63
UGT1A1	Estradiol		ND		4.5	ND			ND		5.4	ND	
UGT1A3	Chenodeoxycholic acid				51						48		
UGT1A4	Trifluoperazine				>100						>100		
UGT1A6	1-Naphthol				52						55		
UGT1A9	Propofol				19						>42		
UGT2B7	Morphine				>100						>100		
UGT2B10	Levomedetomidine				26						41		
UGT2B15	*S*-Oxazepam				38						45		
UGT2B17	Testosterone				7.2						18		

ND: Not determined

Figure 2. IC_{50} plots of CYP1A2, CYP2C8 and UGT1A1 inhibition by oligonucleotides in HLM

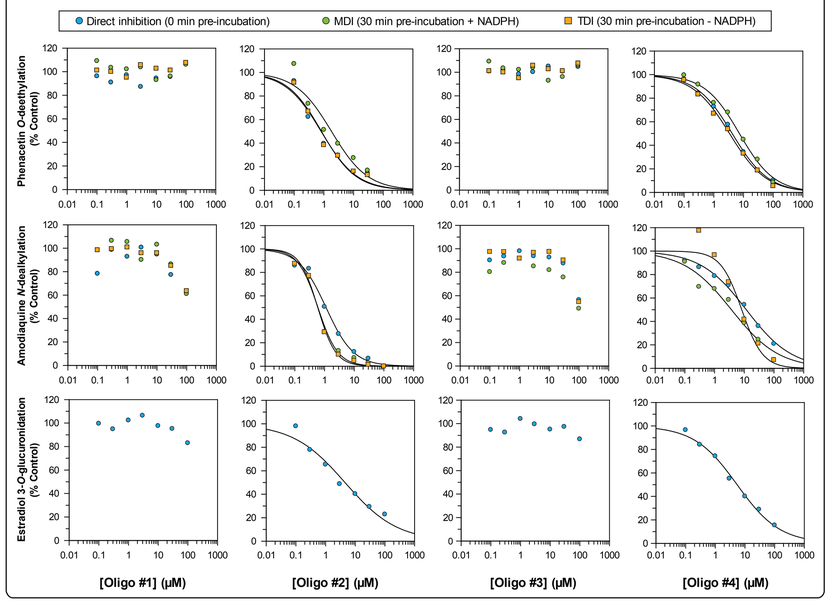


Table 3. IC_{50} values for CYP and UGT inhibition with oligonucleotides in CHH

Enzyme	Substrate	IC_{50} (µM)											
		Oligo #1			Oligo #2			Oligo #3			Oligo #4		
		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									77			
CYP2C8	Amodiaquine					35	44						
CYP2C9	Diclofenac					>100	>100			>100			
CYP2C19	*S*-Mephenytoin							36					
CYP2D6	Dextromethorphan												
CYP3A4/5	Midazolam						49	>100					
UGT	4-Methylumbelliferone						>100						

Table 4. IC_{50} values for uptake and efflux transporter inhibition with oligonucleotides

Transporter	Substrate	IC_{50} (µM)			
		Oligo #1	Oligo #2	Oligo #3	Oligo #4
OATP1B1	Estradiol 17-β-glucuronide	>100	90	>100	>100
OATP1B3	Estradiol 17-β-glucuronide		14		92
OAT1	*p*-Aminohippuric acid	46	12	52	16
OAT3	Estrone 3-sulfate	>100	26	69	>100
OCT1	Tetraethylammonium bromide		>100	>100	
OCT2	Metformin	17	14	27	29
Pgp	Digoxin	>100	>100	>100	>100
BCRP	Prazosin				

Figure 3. IC_{50} plots of CYP1A2, CYP2C8 and UGT inhibition by oligonucleotides in CHH

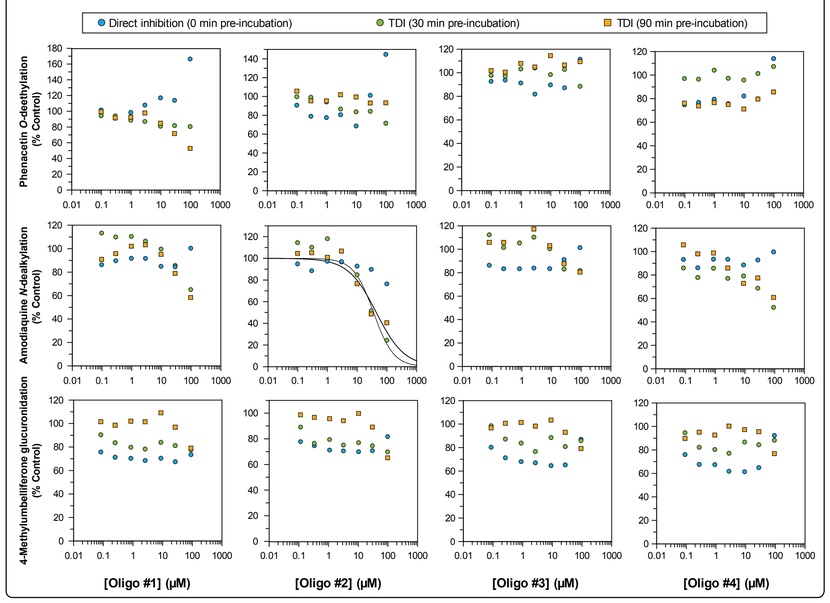
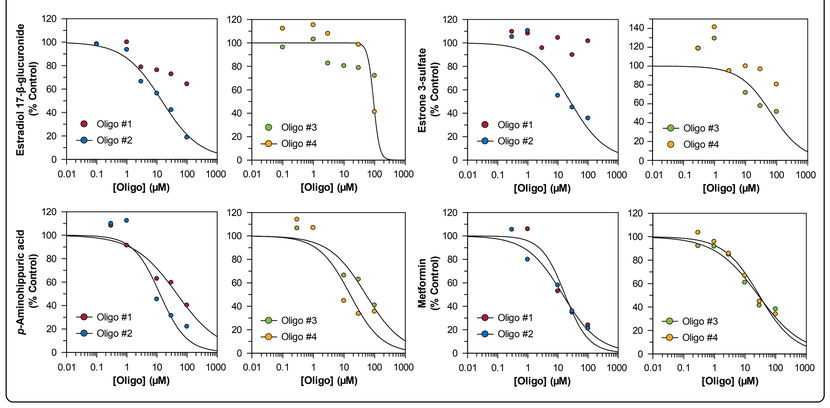


Figure 4. The inhibition of the uptake transporters OATP1B3, OAT1, OAT3 and OCT2 by oligonucleotides



Conclusions

- In HLM, phosphorothioate but not phosphodiester oligonucleotides were found to be inhibitors of CYP and UGT enzymes.
- In CHH, inhibition of CYP and UGT enzymes was dramatically less potent than in HLM, suggesting test system-dependent inhibitory effects.
- Both phosphodiester and phosphothioate oligonucleotides were found to be inhibitors of uptake but not efflux transporters.
- Overall this study has implications on the design of *in vitro* DDI studies for oligonucleotide based therapeutics based upon test system-dependent differences.

References

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