FIGURE 5

Assessment of CYP2C8 time-dependent inhibition by phosphorothioate-containing oligonucleotides in human liver microsomes with IC_{50} shift and ultracentrifugation experiments.

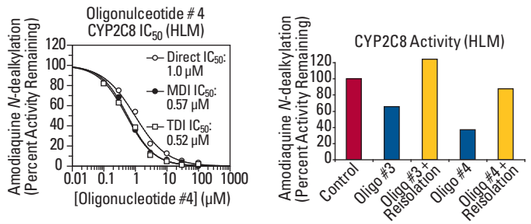

FIGURE 6

System-dependent inhibition of CYP1A2 (a) and CYP2C8 (b) activity by phosphorothioate-containing oligonucleotides in human liver microsomes *versus* human hepatocytes.

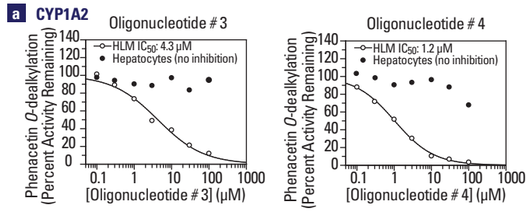

FIGURE 7

System-dependent inhibition of CYP2C8 activity by phosphorothioate-containing oligonucleotides in human hepatocytes incubated in medium *versus* human plasma.

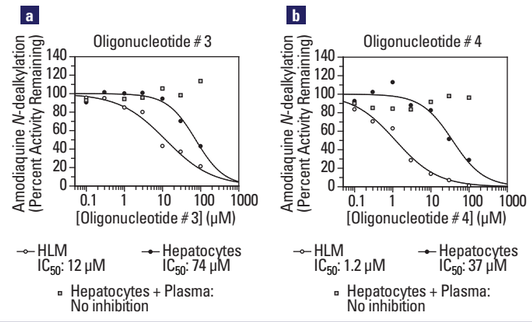

Figure 7 compares the inhibitory potential of phosphorothioate-containing oligonucleotides #3 and #4 in human hepatocytes incubated in medium *versus* human plasma towards CYP2C8. When these oligonucleotides were incubated with hepatocytes in the presence of plasma, no inhibition of CYP2C8 was observed. These data suggest that phosphorothioate-containing oligonucleotides are taken up into hepatocytes (as evidenced by the inhibition of CYP2C8 in hepatocytes incubated in medium), but that these large, polyanionic oligonucleotides bind extensively to plasma protein, which appears to restrict their uptake into hepatocytes and consequently restrict their ability to inhibit CYP2C8.

CONCLUSION

- This study identifies oligonucleotide-based molecules as having the potential to inhibit certain CYP enzymes in human liver microsomes.
- The microsomal enzymes most potently inhibited were CYP1A2 and CYP2C8.
- Oligonucleotides with a phosphorothioate backbone were more potent inhibitors than the corresponding sequence with a phosphodiester backbone.
- The ability of oligonucleotides to inhibit CYP enzymes in human liver microsomes shows some degree of time dependency probably due to the slow rate of onset, a consequence of the repulsion between the negatively charged phospholipid bilayer and the negatively charged oligonucleotide.
- CYP inhibition is apparently due to the oligonucleotide itself, not a low molecular weight contaminant.
- The potency with which oligonucleotides inhibited CYP1A2 and CYP2C8 in liver microsomes decreased markedly when tested in human hepatocytes, and decreased even further when tested in human hepatocytes in the presence of human plasma.
- It is not known whether oligonucleotides can cause clinically significant inhibition of CYP1A2 or CYP2C8.

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Inhibition of Cytochrome P450 (CYP) Enzymes, CYP1A2 and CYP2C8, by Oligonucleotides in Human Liver Microsomes (HLM): A System-Dependent Outcome.

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ABSTRACT

Oligonucleotide-based molecules represent an evolving class of new drug candidates with diverse structures, functions and therapeutic applications. Oligonucleotides are large polyanionic molecules not metabolized by CYP enzymes; accordingly, oligonucleotides would not be expected to inhibit CYP enzymes. Currently, there are no reported cases of clinical interactions of oligonucleotide-based drugs with co-medications cleared primarily by CYP-dependent metabolism. Unexpectedly, in HLM, we observed inhibition of CYP1A2 and CYP2C8 by oligonucleotides. In the present study, four oligonucleotides, two with phosphodiester backbones (oligo's #1 and #2) and two with phosphorothioate backbones (oligo's #3 and #4) were evaluated (10 µM) as direct-acting, time-dependent (TDI) and metabolism-dependent inhibitors of seven human CYP enzymes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4. Little or no CYP inhibition was observed with oligonucleotides containing a phosphodiester backbone (oligo's #1 and #2). However, in HLM, both phosphorothioate oligonucleotides (oligo's #3 and #4) were relatively potent direct inhibitors of CYP1A2 (IC_{50} 4.3 and 1.0 µM, respectively) and CYP2C8 (IC_{50} 12 and 1.2 µM, respectively) and were mixed-type inhibitors of CYP1A2 (K_i values 3.7 and 0.8 µM, respectively). In the case of oligo #4, there was some evidence of TDI (not NADPH-dependent) of CYP2C8 (IC_{50} shift from 1.0 to 0.5 µM), which was reversible after re-isolation of microsomes by ultracentrifugation. Both oligonucleotides were filtered (MW < 3000); the low molecular weight filtrate caused no CYP inhibition, suggesting that CYP inhibition is attributable to the parent molecule. In contrast to HLM, neither phosphorothioate-containing oligonucleotide (oligo's #3 and #4) inhibited CYP1A2 in human hepatocytes (suspensions), although they did cause weak inhibition of CYP2C8 (IC_{50} 74 and 37 µM, respectively). The weak inhibition of CYP2C8 by oligo's #3 and #4 in hepatocytes was further reduced in the presence of human plasma. Although it remains to be determined whether oligonucleotide-based drugs can cause clinically significant inhibition of CYP1A2 and/or CYP2C8, the large difference in inhibitory potency between HLM and human hepatocytes with oligonucleotides provides another example of system-dependent CYP inhibition.

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INTRODUCTION

Oligonucleotides are large, polyanionic molecules that can be advantageously designed with structural modifications for diverse therapeutic applications. Consequently, oligonucleotide-based drugs represent an evolving class of new drug candidates. Typically, oligonucleotides are metabolized by endo- and exonucleases, not by cytochrome P450 (CYP) enzymes; however, recent advances in their backbone chemistry have prolonged half-lives of oligonucleotides by reducing their susceptibility to nuclease hydrolysis. Oligonucleotides are readily taken up into multiple cell types in the liver, including Kupffer and sinusoidal endothelial cells and, to a lesser extent, parenchymal hepatocytes (Geary *et al.*, 2006; Yu *et al.*, 2009). We are not aware of reported cases of clinical drug-drug interactions (DDI) between oligonucleotide-based drugs and co-medications, including drugs cleared primarily by CYP-dependent metabolism (see table below). Accordingly, based on structure, disposition and literature, oligonucleotides would not be expected to inhibit CYP enzymes.

Published Reports of Clinical DDI Studies with Oligonucleotide-based Drugs

In vivo Probe / co-medication	Major Route of Clearance	Clinical Interaction
Simvastatin	CYP3A4	NO[1]
Ezetimibe	Glucuronidation	NO[1]
Rosiglitazone	CYP2C8 > 2C9.	NO[2]
Glipizide	CYP2C9 >2C8	NO[2]
Metformin	Renal	NO[2]
Cisplatin	Renal	NO[3]
Gemcitabine	Nucleoside kinases	NO[3,4]

([1]Yu *et al.*, 2009; [2]Geary *et al.*, 2006; [3]Villalona-Calero *et al.*, 2004; [4]Adjei *et al.*, 2003)

Similar to clinical observations, a recent published report indicates that oligonucleotide-based drugs do not cause *in vitro* CYP inhibition with human hepatocytes (Yu *et al.*, 2009). Unexpectedly, however, we observed CYP inhibition by oligonucleotides in human liver microsomes (HLM). This study describes several experimental approaches (IC_{50} shifts, K_i determinations, ultracentrifugation, *etc.*,) to characterize the potential for oligonucleotides to cause direct, time-dependent (TDI) or metabolism-dependent (MDI) CYP inhibition in two *in vitro* test systems, namely, human liver microsomes (HLM) and human hepatocytes.

MATERIALS AND METHODS

Chemicals: Pooled human liver microsomes (HLM) and cryopreserved human hepatocytes were prepared at XenoTech, LLC (Lenexa, KS). The sources of the reagents used in this study have been described elsewhere (Robertson *et al.*, 2000; Ogilvie *et al.*, 2006; Paris *et al.*, 2009).

Oligonucleotides: Four DNA oligonucleotides were synthesized by IDT DNA Technologies (Coralville, IA). Each of two DNA oligonucleotide sequences, designated alpha (n = 20 bases) and beta (n = 21 bases), were synthesized in two formats: one with a phosphodiester backbone and one with a phosphorothioate backbone. The four oligonucleotides are designated 1-4 as follows:

Oligo #1: alpha sequence (n = 20 bases) with a phosphodiester backbone (MW: 5911.9)
Oligo #2: beta sequence (n = 21 bases) with a phosphodiester backbone (MW: 6459.2)
Oligo #3: alpha sequence (n = 20 bases) with a phosphorothioate backbone (MW: 6217.0)
Oligo #4: beta sequence (n = 21 bases) with a phosphorothioate backbone (MW: 6780.4)

All oligonucleotides were purified by standard desalting, shipped in lyophilized form, reconstituted in 1 mM phosphate-buffered saline and stored at -20° C.

CYP inhibition in HLM: Oligonucleotides were assessed for their potential to cause three types of CYP inhibition in HLM:

1. Direct inhibition, in which case the oligonucleotide and the CYP marker substrate were added simultaneously and incubated for 5 min to determine CYP activity;
2. Time-dependent inhibition (TDI), in which case the oligonucleotide was incubated for 30 min with HLM in the *absence* of NADPH prior to the addition of CYP marker substrate (followed by a 5-min incubation to determine CYP activity);
3. Metabolism-dependent inhibition (MDI) in which case the oligonucleotide was incubated for 30 min with HLM in the *presence* of NADPH prior to the addition of CYP marker substrate (followed by a 5-min incubation to determine CYP activity);

Marker substrate reactions were conducted for 5 min with NADPH-fortified HLM (typically at 0.1 mg/mL; 0.0125 mg/mL for CYP2C8 and 0.05 mg/mL for CYP3A4 midazolam) in a reaction mixture containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4) and an NADPH-generating system (1 mM NADP, 5 mM glucose-6-phosphate, and 1 U/mL glucose-6-phosphate dehydrogenase), at the final concentrations indicated. These reactions were performed at the approximate K_m for each marker reaction: phenacetin (40 µM; CYP1A2), bupropion (50 µM; CYP2B6), amodiaquine (1.5 µM; CYP2C8), diclofenac (7 µM; CYP2C9), *S*-mephenytoin (40 µM; CYP2C19), dextromethorphan (75 µM; CYP2D6), testosterone (70 µM; CYP3A4/5) or midazolam (4 µM; CYP3A4/5). In all cases, marker substrate reactions were terminated by the addition of an equal volume of acetonitrile containing internal standard (deuterated metabolite). Metabolite formation was determined by LC-MS/MS as described previously (Ogilvie *et al.*, 2006; Ogilvie *et al.*, 2008, Paris *et al.*, 2009).

To assess whether CYP inhibition was caused by a low molecular weight contaminant, oligonucleotides (100 µM) were filtered through a 3 kDa micro-centrifuge filter (Millipore; Billerica, MA) and the resultant filtrate was assessed for its ability to inhibit CYP activity in HLM as described above.

To assess reversibility of the observed time-dependent inhibition of CYP activity, the microsomal fraction was isolated by ultracentrifugation (100,000 x *g*; 60 min; 4°C) following a 30 min pre-incubation of the oligonucleotides with HLM. The supernatant fraction was discarded and the microsomal pellet was rinsed with potassium phosphate buffer (50 mM, pH 7.4). The microsomal pellets were resuspended in 250 mM sucrose, after which protein concentration was determined by the Pierce BCA Protein Assay (Pierce, Rockford, IL) and residual CYP activity was assessed with marker substrate reactions as described above.

CYP inhibition in Human Hepatocytes: Oligonucleotides were assessed for their potential to cause inhibition of CYP1A2 or CYP2C8 activity in suspensions of pooled (n = 3) cryopreserved human hepatocytes. Oligonucleotides were pre-incubated with human hepatocytes (0.5 x 10^6 cells/mL) for 30 min at 37 °C in Krebs-Henseleit (KHB) buffer or human plasma. Marker substrate reactions were initiated by the addition of phenacetin (40 µM; CYP1A2) or amodiaquine (7 µM; CYP2C8) and terminated after 15 min by the addition of an equal volume of acetonitrile containing internal standard (deuterated metabolite). Metabolite formation was determined by LC-MS/MS as described previously (Ogilvie *et al.*, 2006, Ogilvie *et al.*, 2008, Paris *et al.*, 2009).

RESULTS

Figure 1 shows the results of experiments to examine the ability of oligo's #1 – #4 to function as direct inhibitors of CYP enzymes in HLM. The two phosphodiester oligo's (#1 and #2) caused little or no inhibition of the CYP enzymes examined. In contrast, the two phosphorothioate oligo's (#3 and #4) caused direct inhibition of several CYP enzymes, most notably CYP1A2 (75 and 91%, respectively) and CYP2C8 (45 and 92%, respectively).

Figure 2 shows the effects of ultrafiltration on the ability of oligo's #3 and #4 to inhibit CYP1A2 and CYP2C8. Prior to filtration through a 3-kDa filter, these two phosphorothioate oligo's caused substantial direct inhibition of CYP1A2 and CYP2C8. However, neither enzyme was inhibited by the low molecular weight filtrates from these oligo's. These data suggest that the ability of the oligo's #3 and #4 to inhibit CYP1A2 and CYP2C8 is likely attributable to the parent phosphorothioate oligonucleotide and not to a low molecular weight contaminant or degradation product.

Figure 3 shows that the phosphorothioate oligo's (#3 and #4) were both mixed inhibitors of microsomal CYP1A2 and CYP2C8 (*i.e.*, they inhibited these enzymes by a combination of competitive and non-competitive inhibition). Oligo's #3 and #4 inhibited CYP1A2 with a K_i of 3.7 and 0.8 µM, and they inhibited CYP2C8 with an IC_{50} of 12 and 1.2 µM, respectively. CYP1A2 and CYP2C8 were more potently inhibited by oligo #4 than oligo #3, suggesting that sequence, and possibly secondary structure, are determinants of microsomal CYP inhibition potential by phosphorothioate-containing oligonucleotides.

Figure 4 shows an assessment of the potential of the phosphorothioate oligonucleotides to cause time-dependent inhibition (TDI) or metabolism-dependent inhibition (MDI) of CYP enzymes in HLM. Oligo's #3 and #4 caused modest time-dependent inhibition of CYP2B6, CYP2C8 and CYP2C9 inasmuch as their inhibitory potency increased following a 30-min pre-incubation of the oligos with HLM in the absence of NAPDH.

Figure 5 shows that phosphorothioate oligo #4 caused time-dependent inhibition of CYP2C8 activity (the IC_{50} value shifted from 1.0 to 0.52 µM), and this effect was reversible as determined by ultracentrifugation (*i.e.*, following the re-isolation of microsomes to remove any oligonucleotide that was not bound to CYP2C8). These results suggest that the time-dependent inhibition of microsomal CYP activity is due to the phosphorothioate-containing oligonucleotides having a "slow on-rate" or impaired diffusion due to interactions between the microsomal phospholipid bilayer and the large, poly-anionic oligonucleotides (both of which are extensively negatively charged).

Figure 6 compares the inhibitory potential of phosphorothioate-containing oligonucleotides #3 and #4 in HLM *versus* human hepatocytes (in medium) towards CYP1A2 and CYP2C8. In contrast to their inhibitory effect in HLM, oligo's #3 and #4) caused little or no inhibition of CYP1A2 in human hepatocytes. In the case of CYP2C8, both of these phosphorothioate oligonucleotides caused modest inhibition of CYP2C8 in human hepatocytes incubated in medium (IC_{50}: 74 and 37 µM, respectively); however, the inhibition observed in hepatocytes was considerably less than that in HLM (IC_{50}: 12 and 1.2 µM, respectively).

FIGURE 1

Comparison of direct inhibition of CYP enzymes in human liver microsomes by oligonucleotides containing a phosphodiester backbone (oligo's #1 and #2) or phosphorothioate backbone (oligo's #3 and #4).

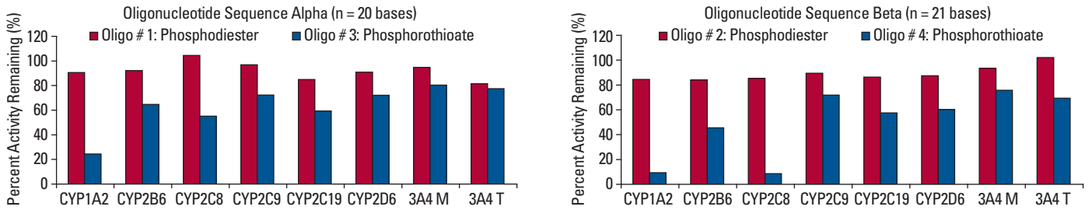


FIGURE 2

The effects of ultrafiltration on the ability of phosphorothioate-containing oligonucleotides to inhibit microsomal CYP activity.

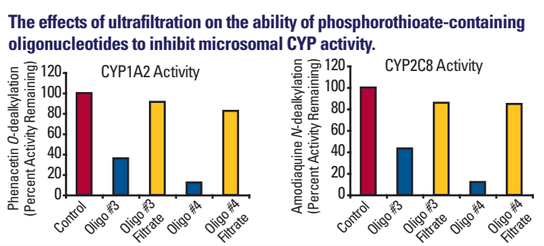
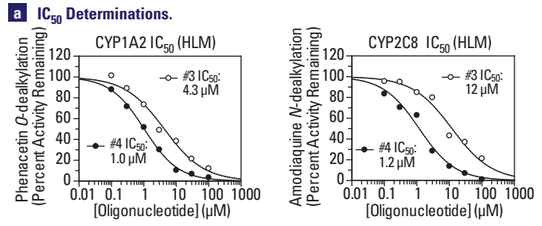


FIGURE 3

The effects of ultrafiltration on the ability of phosphorothioate-containing oligonucleotides to inhibit microsomal CYP activity.

a **IC_{50} Determinations.**



b **K_i Determinations.**

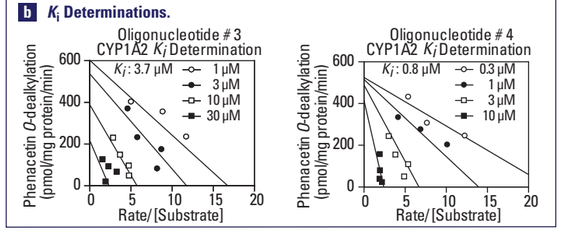


FIGURE 4

Assessment of time-dependent inhibition of multiple CYP activities by phosphorothioate-containing oligonucleotides in human liver microsomes.