PHOSPHORODIAMIDATE MORPHOLINO ANTISENSE OLIGOMERS INHIBIT EXPRESSION OF HUMAN CYTOCHROME P450 3A4 AND ALTER SELECTED DRUG METABOLISM

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ABSTRACT:

Antisense phosphorodiamidate morpholino oligomers (PMO) inhibit gene expression by preventing ribosomal assembly, thus preventing translation. Inhibition of cytochrome P450 (P450) 3A4 expression was examined in primary human hepatocytes from 11 donors and in Caco-2 cells (stably transfected with CYP3A4 cDNA on an extrachromosomal vector) by evaluating the metabolism of substrate 7-benzyloxy-4-[trifluoromethyl]-coumarin and Western immunoblot analysis. Cellular uptake of PMO was confirmed in both cell systems using fluorescein-labeled PMOs. Three antisense PMO sequences and two control PMO sequences were tested. AVI-4557, a 20-mer PMO with the sequence 5'–CTGGGATGAGACCATC3' was selected as the optimal agent. AVI-4557 inhibited expression of CYP3A4 in Caco-2/h3A4 cells by 64% at 24 h following administration of 2.8 μM by an assisted delivery protocol. Inhibition of CYP3A activity was observed in primary human hepatocytes after 24 h exposure to AVI-4557 by an average of 32 ± 11%. Furthermore, AVI-4557 exposure resulted in a sequence-dependent inhibition of cyclophosphamide-related cytoxic activity and a sequence-dependent induction of paclitaxel-related cytoxic activity in both cell types. Finally, the cytoxic activity of cisplatin was not affected with AVI-4557 treatment in either cell type. These studies indicate AVI-4557 is an effective and specific inhibitor of CYP3A4 expression.

Cytochromes P450 (P4503A4) are heme-containing monoxygenases that metabolize thousands of compounds, including xenobiotics and endogenous ligands. CYP3A is the principle P450 subfamily that is responsible for phase I metabolism of about 60% of clinically relevant drugs (Guengerich, 1999). CYP3A4 is the predominant isofrom of CYP3A in adult humans and can catalyze numerous metabolic processes including aliphatic oxidation, aromatic hydroxylation, N-dealkylation, O-demethylation, S-demethylation, oxidative deamination, sulfoxide formation, N-oxidation, and N-hydroxylation (Dresser et al., 2000; Wrighton et al., 2000). These reactions can participate in the activation, inactivation, and/or elimination of the substrate pharmacueticals, thereby directly influencing their pharmacokinetic and pharmacodynamic profile (Benet et al., 1996; Thummel and Wilkinson, 1998).

The current study uses an antisense approach for inhibition of CYP3A4 in two distinct model systems: primary cultures of human hepatocytes (applications reviewed by Cross and Bayliss, 2000) and the human colon carcinoma cell line Caco-2 stably transfected with CYP3A4 cDNA (described by Crespi et al., 1996). This approach was employed to test the hypothesis that inhibition of CYP3A4 activity would alter the cytotoxicity of two commonly used chemotherapeutic agents, paclitaxel and cyclophosphamide, but have no effect on cisplatin toxicity.

The chemistry of the antisense agents used in this study was phosphorodiamidate morpholino oligomer (PMO) (reviewed by Summerton and Weller, 1997a; Summerton, 1999; Iversen, 2001a,b). PMOs have been previously shown to have high efficacy in vivo (Qin et al., 2000), particularly for targets in the liver following intraperitoneal (Arora and Iversen, 2000a,b) and oral administration (Arora et al., 2002). This approach to CYP3A4 inhibition by antisense PMO represents a potential strategy (reviewed by Arora and Iversen, 2001) for altering pharmacokinetics and pharmacodynamics of multiple, clinically relevant drugs metabolized by this enzyme.

Materials and Methods

Chemicals. Cyclophosphamide monohydrate (cyclophosphamide), cis-platinum(II)-diamine dichloride (cisplatinum), and paclitaxel were all purchased from Sigma-Aldrich (St. Louis, MO). All phosphodiester DNA oligomers used as delivery agents for PMOs were purchased from Integrated DNA Technologies Inc. (Coralville, IA).

Cells and Cell Culture. Caco-2 cells stably transfected with human CYP3A4 cDNA on an extrachromosomal vector p220CMV3A4 were purchased from Gentest Corp. (Woburn, MA) and cultured in 75-mm² flasks using Dulbecco’s modified Eagle’s medium supplemented with 1× minimum Eagle’s medium nonessential amino acids, 25 mM HEPES, 8.5 mg/ml glucose, and 15% fetal bovine serum. This cell line has been previously described (Crespi et al., 1996). Freshly plated (not frozen) primary human hepatocytes...
from 11 donors were purchased over a period of 9 months from In Vitro Technologies (Baltimore, MD) or Tissue Transformation Technologies (Edison, NJ) and were cultured using their proprietary serum-free media.

**PMO Synthesis.** All PMOs were synthesized at AVI BioPharma (Corvallis, OR) as previously described (Summerton and Weller, 1997b). Purity was greater than 90% full-length as determined by reverse phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Three antisense PMOs were designed to target human CYP3A4 and were named 1-0-556 (19-mer), AVI-4557 (20-mer), and 1-0-558 (21-mer). All three PMOs are complementary to the translation initiation region and encompass the AUG start site based on a previously published sequence (GenBank accession number M14096). The base sequence of 1-0-556 is 5'-CTGGGATGAGAGCCATCACT-3'. The base sequence of AVI-4557 is 5'-CTGGGATGAGGGCCATCACT-3'. The base sequence of 1-0-558 is 5'-GCTGGGATGAGGGCCATCACT-3'. The control PMO used in these studies is antisense to the AUG translation initiation region of the rat orthologs gene CYP3A2 mRNA (GenBank accession number U09742) and has multiple mismatches with the human CYP3A4 sequence. The control PMO is named AVI-4472, and its base sequence is 5'-GAGCTGAAAGCAGGTC-3'.

**Assisted PMO Delivery.** The PMOs were delivered into cells at a concentration of 1.4 or 2.8 μM according to the following protocol modified from previously published Gene Tools’ Special Delivery (SD) Protocol (Morcos, 2001; Gene Tools Technical Bulletin 05/23/2000, Corvallis, OR). Partially complementary DNA molecules with a 10-base adenine 5’ overhang were synthesized to serve as carriers for each PMO sequence. The partially complementary DNA used for delivery of antisense PMO AVI-4557 was 5’-AAAAAAAAGTGATGGCTCTCATC-3’. The partially complementary DNA used for delivery of control PMO AVI-4472 was 5’-AAAAAAAAGGGATGGACCTGCTTTCA-3’. A PMO/DNA duplex was formed by incubating 1 μM stocks of the PMO and the respective partially complementary DNA in a 3:2 ratio for 10 min at room temperature. For the 1.4 μM treatment mixture, 9.3 μl of the duplex stock was diluted with 200-μl volume in deionized water. Ten microliters of the weakly basic delivery reagent ethoxylated polyethylenimine (Gene Tools) was added. The tube was vortexed and incubated for 20 min at room temperature. Serum-free media (3.6 ml) and deionized water (180.7 μl) were added to bring the final volume to 4.0 ml. Subconfluent-cultured cells were exposed to the above mixture for 3 h at 37°C (1.5 ml/well for 6-well plate and 150 μl/well for 96-well plate). This mixture was then aspirated and replaced with normal serum-containing media. Cells were harvested for CYP3A analysis following 24 h.

**CYP3A Enzyme Activity Assay.** This assay was modified from Gentest Technical Bulletin, version 3, dated 09/25/1998 (Gentest Corp.) and is a tool for determining CYP3A activity (Stresser et al., 2000). A 100-μg aliquot of the S9 fraction was diluted in 0.1 M potassium phosphate (Sigma-Aldrich) to a volume of 500 μl. This was followed by addition of 15 μl of 25 mM (in acetonitrile) substrate 7-benzoxyl-4-[trifluoromethyl]-coumarin (HFC), at the excitation wavelength of 409 nm and emission wavelength of 530 nm. All readings were compared with a standard curve prepared from 5 μM stock solution (in acetonitrile) of HFC (Gentest Corp.). Enzyme activities in primary human hepatocytes were typically measured on day 4 following plating.

**Photomicroscopy.** Cells were washed three times with phosphate-buffered saline and examined under a Nikon Diaphot 300 microscope (Nikon Instruments, Melville, NY) connected to an Olympus Magnafire SP-brand digital camera (Olympus America Inc., Melville, NY). The exposure times were kept constant for all fluorescent pictures at 8 s.

**Immunoblot Analysis.** Levels of CYP3A4 and β-actin proteins were determined from Western immunoblots in liver S9 fractions. Total protein (30 μg) was separated on a 12% sodium dodecylsulfate/acylamide gel and immunoblotted according to standard techniques as described before (Arora and Iversen, 2000b). Polyclonal primary antibodies for CYP3A4 were from Gentest Corp., and primary monoclonal antibodies for β-actin (clone AC-40) were from Sigma-Aldrich. β-actin immunodetection was performed to confirm that all lanes were loaded with similar amounts of protein. Densitometry was performed on a Kodak Image Station 440 (PerkinElmer Life Sciences, Boston, MA).

**Cell Viability Assay.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) assay was used to measure cell viability. One-hundred microliters of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well in a 96-well plate at 37°C till blue coloration started to appear in the cells. The medium was then aspirated and replaced with dimethyl sulfoxide. The absorbance was measured at 540 nm on a Softmax multi-well plate reader (Molecular Devices Corp., Sunnyvale, CA).

**Statistics.** All statistical calculations and graph plots were done on Prism version 3.0 software (GraphPad, San Diego, CA). Data were analyzed by calculating two-tailed p values using unpaired t tests. Statistical significance was denoted by * for p < 0.05 and ** for p < 0.005.

**Results**

**PMO Delivery in Cells by SD Protocol.** Cellular uptake studies were done both in Caco-2/h3A4 and primary human hepatocytes with a 20-mer PMO labeled with carboxy-fluorescein on the 5′ end (F-PMO). F-PMO uptake was observed in both cell types with the SD protocol. Following the SD protocol for F-PMO delivery (see Materials and Methods section), cell monolayers were rinsed several times with phosphate-buffered saline to remove any fluorescence resulting from extraneous F-PMO. The delivery of the F-PMO was confirmed by examination of the monolayers under a phase contrast microscope in fluorescence mode (Fig. 1A and B). The SD protocol was designed to deliver PMOs at concentrations of 1.4 or 2.8 μM. Clear evidence of delivery of F-PMO was observed at both doses. The higher dose produced more intense signal. The pattern of distribution of the F-PMO was found to be diffused in the cytoplasm and relatively more intense in the nuclei both in Caco-2/h3A4 and primary human hepatocytes. The replacement of F-PMO with unlabeled PMO in these uptake studies resulted in completely dark fields under identical fluorescence microscopy conditions (data not shown).

**Unassisted PMO Delivery in Cells.** Unassisted F-PMO uptake
was observed only in primary human hepatocytes following a 24-h incubation at concentrations of 30 μM and above (Fig. 1, C and D). The cellular distribution pattern was essentially similar to that observed with the SD protocol. The fluorescence intensity, however, was markedly lower with 30 μM unassisted delivery when compared with 2.8 μM assisted delivery under identical photography conditions. The replacement of F-PMO with unlabeled PMO resulted in completely dark fields under identical fluorescence microscopy conditions (data not shown). Lastly, unassisted F-PMO uptake was not observed in Caco-2/h3A4 cells.

Selection of the Optimal CYP3A4 Inhibitory Agent. Three antisense PMO agents were tested for inhibition of BFC hydroxylation activity in both model systems: Caco-2/h3A4 cells and primary human hepatocytes. The three antisense agents were a 19-mer (1-0-556), 20-mer (AVI-4557), and a 21-mer (1-0-558). Two control PMOs were used in all studies in addition to vehicle controls: a 20-mer c-myc antisense (AVI-4126) and a 22-mer rat CYP3A2 antisense (AVI-4472). The baseline BFC hydroxylation activity in Caco-2/h3A4 cell S9 fraction was determined to be 1558 ± 47 pmol HFC/100 μg S9/h (Fig. 2A). Increasing amounts of vehicle, AVI-4126 or AVI-4472, did not have any statistically significant effect on BFC hydroxylation activity. All of the three test PMO antisense agents designed to target human CYP3A4 showed decrease in BFC hydroxylation activity at both doses, with the 19-mer showing 50% reduction at 2.8 μM compared with 64% by the 20-mer and 60% by the 21-mer.

To rule out toxicity issues related to the SD delivery technique as well generate a dose-response curve with multiple doses, further studies were performed in Caco-2/h3A4 cells using the scrape-loading technique for delivery of PMO agents (see Materials and Methods for description of technique). This technique allowed for testing of multiple PMO doses, unlike the SD technique, which restricted the doses to 1.4 and 2.8 μM. A dose-response with 1, 5, 10, 15, 20, and 30 μM was constructed using this technique (Fig. 2B). Increasing amounts of vehicle or control PMOs AVI-4126 and AVI-4472 did not have any statistically significant effect on BFC hydroxylation activity. The 19-mer and 21-mer antisense agents both showed maximal inhibition at 30 μM, decreasing enzyme activity by 53 ± 6% (p < 0.005) and 65 ± 5% (p < 0.005), respectively. The 20-mer antisense PMO showed maximal inhibition of 60 ± 8% (p < 0.005) at 20 μM, with a plateau thereafter.

Similar efficacy data were also generated in primary human hepatocytes from donor 5 (see Table 1 for description of donor characteristics and partial data). The baseline activity was determined to be 587 pmol HFC/100 μg S9/h. There was a reduction in enzyme activity following SD delivery of all three antisense PMOs at both doses. At 2.8 μM, the reductions in enzyme activity were 31, 39, and 37% for the 19-mer, 20-mer, and 21-mer antisense PMOs, respectively.

The 20-mer PMO, AVI-4557, was determined to be the optimal CYP3A4 antisense agent based on above data and issues related with ease of synthesis.

Inhibition of CYP3A4 Enzyme Activity in Primary Human Hepatocytes. CYP3A4 enzyme activity was assessed by dehydroxylation of BFC, which generates the fluorescent product HFC. This assay has been described previously as an accurate and sensitive marker of CYP3A activity (Stresser et al., 2000). The enzyme activity from all 11 primary hepatocyte donors is described in Table 1. These primary hepatocytes were purchased as freshly plated monolayers over a period of 9 months (see Materials and Methods section for details). The baseline activity varied from 431 to 847 nmol HFC/100 μg S9/h, with the exception of donor 4. The enzyme activity of donor 4 (1 year-old infant male) was 223 nmol HFC/100 μg S9/h. Treatment of hepatocytes by 2.8 μM AVI-4557 (assisted delivery by SD protocol) for 24 h caused reduction of enzyme activity in cells from every donor. The extent of reduction varied from 11 to 47%. The least reduction was observed in cells from donor 10, a 36-year-old white female on unknown allergy medications. The maximal reduction was observed in donor 9, a 49-year-old white male (Table 1).

Pentoxyresorufin O-dealkylation activity was measured in all treatment groups from 6 of 11 hepatocyte donors as a control for antisense PMO specificity. No apparent change in CYP2B activity was observed between treatment groups in any individual (data not presented).

Immunoblot Verification of CYP3A4 Reduction following AVI-4557 Treatment. S9 fractions of Caco-2/h3A4 cells as well as all 11 primary human hepatocyte lots were analyzed by Western immunoblot following treatments with vehicle, control PMO AVI-4472, and antisense PMO AVI-4557 using SD protocol at 2.8 μM. A total of 20 μg total S9 protein was run on 10% SDS-polyacrylamide gel electrophoresis gels. The primary antibody used to probe the blots was MAB-3A4, a monoclonal CYP3A4 antibody purchased from Gentest Corp. Representative data from donors 1 and 2 are presented in Fig. 3. Primary hepatocyte S9 fractions from all donors, with one exception, showed a doublet of bands with the major band on top at approximately 57 kDa. The exception was donor 4 (see Table 1 for description). No bands were detected from hepatocyte S9 fractions of this donor, a 1-year-old infant. In a typical Western immunoblot, subtle to no decrease was observed with the control PMO, AVI-4472, whereas a pronounced decrease in band intensity was observed with the antisense PMO, AVI-4557. Following detection of CYP3A4, all blots were stripped and re-probed for β-actin to confirm uniform loading of gels (Fig. 3).

Cytotoxicity Changes of Drugs Metabolized by CYP3A4 in Presence of AVI-4557. Cell viability of Caco-2/h3A4 cells was
studied following cotreatment with PMOs and selected cytotoxic drugs. Cells were pretreated using SD protocol with vehicle, 2.8 \( \mu \)M control PMO AVI-4472, or 2.8 \( \mu \)M antisense PMO AVI-4557, 24 h prior to addition of cytotoxic drugs, as indicated. Cell viability was determined after additional 24-h incubation with increasing concentrations of paclitaxel, cyclophosphamide, or cisplatin (Fig. 4, A–C).

When PMO-pretreated Caco-2/h3A4 cells were cotreated with 0.25 through 5.0 \( \mu \)M paclitaxel, viability of cells pretreated with control PMO or vehicle was not affected in a statistically significant manner with this suboptimal dose. However, cells pretreated with AVI-4557 showed dose-dependent decrease in cell viability (Fig. 4A). Essentially, inverse data were obtained when PMO-pretreated Caco-2/h3A4 cells were cotreated with 50 through 600 \( \mu \)M cyclophosphamide. In this case, viability of cells pretreated with antisense PMO was not affected in a statistically significant manner. However, cells pretreated with vehicle/control PMO showed dose-dependent decrease in cell viability with increasing doses of cyclophosphamide (Fig. 4B). Finally, a decrease in cell viability following increasing doses of cisplatin was not affected by pretreatment with any PMO (Fig. 4C).

Similar changes in cytoidal activity of paclitaxel, cyclophosphamide, and cisplatin were studied in primary human hepatocytes (donor 5) following PMO-pretreatment at 2.8 \( \mu \)M using SD protocol (Fig. 5).

### Table 1

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Race</th>
<th>Medications</th>
<th>Other Details</th>
<th>Vehicle S9</th>
<th>Control PMO S9</th>
<th>Antisense S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>M</td>
<td>White</td>
<td>Tobacco, alcohol</td>
<td>NA</td>
<td>587</td>
<td>501 (14.6%)</td>
<td>354 (39.7%)</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>M</td>
<td>Black</td>
<td>Alcohol, marijuana, benzodiazepine, Hypercholesteremia</td>
<td>2.8 ( \mu )M</td>
<td>671</td>
<td>632 (5.8%)</td>
<td>362 (46.1%)</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>F</td>
<td>White</td>
<td>Lipitor</td>
<td>Hypercholesteremia</td>
<td>847</td>
<td>864 (2.0%)</td>
<td>573 (32.3%)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>M</td>
<td>Black</td>
<td>None</td>
<td>2.8 ( \mu )M</td>
<td>223</td>
<td>199 (10.8%)</td>
<td>189 (15.2%)</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>M</td>
<td>Hispanic</td>
<td>Alcohol, seizures</td>
<td>2.8 ( \mu )M</td>
<td>620</td>
<td>600 (3.2%)</td>
<td>403 (35.0%)</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>F</td>
<td>White</td>
<td>Non-insulin dependent diabetes</td>
<td>2.8 ( \mu )M</td>
<td>431</td>
<td>425 (1.4%)</td>
<td>301 (30.2%)</td>
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<tr>
<td>7</td>
<td>71</td>
<td>F</td>
<td>White</td>
<td>Xanax, Lipitor, Lopressor, Fosamax</td>
<td>2.8 ( \mu )M</td>
<td>702</td>
<td>681 (2.9%)</td>
<td>532 (24.2%)</td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>M</td>
<td>White</td>
<td>None</td>
<td>2.8 ( \mu )M</td>
<td>498</td>
<td>520 (4.4%)</td>
<td>302 (39.4%)</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>M</td>
<td>White</td>
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<td>2.8 ( \mu )M</td>
<td>547</td>
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<td>288 (47.3%)</td>
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<td>10</td>
<td>36</td>
<td>F</td>
<td>White</td>
<td>Allergy medications</td>
<td>2.8 ( \mu )M</td>
<td>604</td>
<td>584 (3.3%)</td>
<td>540 (10.6%)</td>
</tr>
<tr>
<td>11</td>
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<td>M</td>
<td>White</td>
<td>Tobacco, hypertension</td>
<td>2.8 ( \mu )M</td>
<td>482</td>
<td>492 (2.1%)</td>
<td>284 (41.1%)</td>
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</table>

NA, data not available.

**Fig. 3.** Western immunoblot analysis of CYP3A4 protein levels in S9 fractions of primary human hepatocytes from two donors 24 h following SD protocol treatment with vehicle (EPEI), 2.8 \( \mu \)M control PMO AVI-4472, or 2.8 \( \mu \)M antisense PMO AVI-4557, as indicated. **β**-Actin levels were also determined in the same samples to confirm specificity of antisense activity. Individuals 1 and 2 were 56 and 64 year-old white males, respectively. EPEI, ethoxylated polyethyleneimine.
or cisplatin (panel C).

with increasing concentrations of paclitaxel (panel A), cyclophosphamide (panel B), drugs, as indicated. Cell viability was determined after an additional 24-h incubation with 5 μM paclitaxel, 600 μM cyclophosphamide, or 7 μM cisplatin. **, p < 0.005. EPEI, ethoxylated polyethyleneimine.

Due to limited availability of primary human hepatocytes from a single donor, only one dose of coadministered cytotoxic drugs was studied. Coadministration of 5 μM paclitaxel decreased cell viability by 43.9 ± 5.2% (p < 0.005) in the antisense group, 600 μM cyclophosphamide increased cell viability by 39.1 ± 2.1% (p < 0.005) in the antisense group, and cisplatin had no effect on cell viability with any PMO-pretreatment.

Discussion

CYP3A is perhaps the most important and widely implicated enzyme in the arena of drug metabolism and disposition, making it an attractive target for therapeutic manipulation. In the present studies, we have presented data demonstrating the inhibition of the major human isoform of this enzyme, CYP3A4, by use of antisense PMOs. CYP3A4 inhibition was demonstrated in two distinct model systems, namely primary human hepatocytes from 11 donors and Caco-2 cells stably transfected with human CYP3A4 cDNA on an extrachromosomal vector. The uptake of PMOs was confirmed in both model systems at the outset of studies (Fig. 1) and multiple control/antisense sequences were tested (Fig. 2). Consistent CYP3A4 inhibition was demonstrated in a dose-dependent and sequence-dependent manner in both model systems (Table 1 and Fig. 3). Finally, a functional utility of this inhibitory approach was presented by way of altering the cytoidal activity of two substrate drugs, paclitaxel and cyclophosphamide, in a predictable manner (Figs. 4 and 5).

CYP3A4 makes a suitable target for use with antisense technology for various reasons. Its predominant expression in the liver is ideally suited with respect to extensive biodistribution of various oligomer chemistries following in vivo administration (Levin, 1999; Geary et al., 2001; Iversen, 2001b). Furthermore, previous studies have clearly linked the relationship between the P450 levels of an individual and the pharmacokinetics, toxicity, and efficacy of substrate drugs (Collins, 2000; Hirth et al., 2000).

A wide variety of endogenous and exogenous inhibitors of CYP3A4 activity have been described (reviewed by Dresser et al., 2000). The most well studied irreversible inhibitors include the furanocoumarins in grapefruit juice. Reversible inhibitors include macrolide antibiotics, azole antifungals, first generation human immunodeficiency virus protease inhibitors, and cyclosporin. Although relatively modest inhibition of CYP3A4 enzyme activity has been reported in the current studies by use of antisense PMO (32.8 ± 11.9%), the full utility of this approach will not be clear till in vivo studies are completed using pharmacokinetic data of substrate drugs.

Our laboratory has previously reported approximately 50% reduction in midazolam sleep time from 22.4 ± 1.5 min with use of antisense phosphorothioate oligonucleotides targeted to rat CYP3A2 (Desjardins and Iversen, 1995). Of course, the rat CYP3A2 and human CYP3A4 mRNA sequences are quite different at the translation initiation region, in which PMOs are considered to have the maximal antisense activity. The rat CYP3A2 antisense PMO was, in fact, used as an inactive control in the current studies. An important distinguishing feature of the antisense approach versus chemical approach is the potential ability to distinguish between multiple isoforms of the same enzyme.

The comprehensive data detailing inhibition of CYP3A4 activity in primary human hepatocytes from 11 donors (Table 1) highlights the fairly consistent nature of the antisense PMO approach. The average inhibition observed in the antisense group with all donors pooled
together was 32.8 ± 11.9%. The average inhibition observed in the control PMO group with all donors pooled together was 2.8 ± 5.8%. The least inhibition in the antisense group was observed in donors 3 and 10. Donor 3 was a 1 year-old male and likely did not express large amounts of the 3A4 enzyme isoform. Donor 10 was a 36 year-old female who was on unknown allergy medications. It is not clear if these medications may have played a role in the low enzyme inhibition observed in the antisense group (10.6%). The differential nature of CYP3A enzyme activity inhibition between donor samples presented in Table 1 could possibly result from polymorphisms of the target gene. Dai et al. (2001) have recently reported single nucleotide polymorphisms (SNP) in CYP3A4 by direct sequencing of genomic DNA in 72 individuals from three different ethnic groups, including white, black (African Americans and African Pygmies), and Asian peoples. Racial variability was observed for the frequency of the 28 SNPs. Dai et al. have further reported those SNPs that cause coding changes may increase or decrease CYP3A4 enzyme activity. More elaborate studies will be needed to correlate various CYP3A4 polymorphisms and the inhibitory activity of AVI-4557, which is targeted to the translation initiation region of the mRNA.

The cellular uptake of oligomers is a critical issue that needs to be addressed in any antisense study. We observed convincing uptake of F-PMO with assisted delivery techniques in both cell types in this study (Fig. 1). Additionally, unassisted F-PMO uptake was observed in the primary human hepatocytes at higher doses (Fig. 1). It is interesting to note that the cellular distribution pattern of F-PMOs was similar following either uptake mechanism. We observed diffused cytoplasmic signal and a more intense nuclear signal. The decrease in CYP3A4 enzyme activity observed with 30 µM unassisted delivery was typically similar to that observed with 2.8 µM assisted SD technique (data not presented). The unassisted uptake of PMO by primary hepatocytes also correlates well with recent studies published by our group regarding unassisted PMO uptake in liver cells in vivo following oral or intravenous administration in rats (Arora et al., 2002).

The alteration in cytocidal activities of CYP3A4-substrate drugs paclitaxel and cyclophosphamide, when coadministered with AVI-4557, are clear examples of potential utility of this antisense approach. Cyclophosphamide requires CYP3A4-mediated 4-hydroxylation for activation of its cytocidal activity (Roy et al., 1999). Our data indicate that antisense-pretreated Caco-2/h3A4 cells and primary hepatocytes showed no effect on viability in presence of increasing cyclophosphamide concentrations, as opposed to vehicle/control PMO-pretreated cells (Figs. 4B and 5). Unlike cyclophosphamide, paclitaxel does not require activation. Paclitaxel is hydrolyzed by CYP3A4 to more polar and relatively inactive metabolites, such as 3-hydroxy-paclitaxel (C3’

References


