

DMD #3475R

**AUTOMATED SCREENING WITH CONFIRMATION OF MECHANISM-BASED
INACTIVATION OF CYP3A4, CYP2C9, CYP2C19, CYP2D6 AND CYP1A2 IN POOLED
HUMAN LIVER MICROSOMES**

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DMD #3475R

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Abbreviations: DDI, drug-drug interactions; APR, apparent partition ratio; MI, metabolite-intermediate;

MBI, mechanism-based inactivator; CYP450, cytochrome P450; LC/MS/MS, liquid

chromatography/tandem mass spectrometry; HLM, human liver microsomes

DMD #3475R

ABSTRACT

A strategy is proposed to profile compounds for mechanism-based inactivation of CYP3A4, CYP2C19, CYP2C9, CYP2D6 and CYP1A2 based on an apparent partition ratio screen. Potent positives from the screen are confirmed by time- and concentration-dependent inactivation assays. Quasi-irreversible inhibitions are then differentiated from irreversible inactivations by oxidation with potassium ferricyanide and/or dialysis. The three-step screening procedure has been validated with acceptable accuracy and precision for detection and confirmation of mechanism-based inactivators in drug discovery. We report here the apparent partition ratios for 19 mechanism-based inactivators and 4 quasi-irreversible inhibitors obtained under the same experimental conditions. The apparent partition ratio screen was automated to provide throughput for determining structure-mechanism-based inactivation relationships. Information about reversibility can be used to assess potential toxicity mediated by covalent adducts, as well as the potential for pharmacokinetic drug-drug interactions. Direct comparison of known mechanism-based inactivators and quasi-irreversible inhibitors, based on our screening of apparent partition ratios, has identified ritonavir, mibefradil and azamulin as highly effective mechanism-based inactivators; e.g., 1 mole of CYP3A4 was inactivated on turnover of about 2 moles of compound. Other mechanism-based inactivators we identified include bergamottin (CYP1A2 besides previously reported CYP3A4), troglitazone (CYP3A4), rosiglitazone (CYP3A4), and pioglitazone (CYP3A4). Comparison of the apparent partition ratios and inactivation clearance data for the three glitazones suggests that the chromane moiety on troglitazone contributes to its greater potency for mechanism-based inactivation.

Introduction

Reports of adverse, sometimes fatal, events in patients on multiple drug therapies are increasing. A significant number of those events are due to elevated concentrations of drugs in plasma, resulting from pharmacokinetic drug-drug interactions (DDI) that occur because cytochrome P450 (CYP450)-mediated drug metabolism is inhibited. Reports of fatal DDI were responsible for the recent withdrawal of terfenadine (Monahan, 1990), astemizole (Desager and Horsmans, 1995), cisapride (Wysowski and Bacsanyi, 1996), mibefradil (Krayenbuhl et al., 1999) and cerivastatin (Sica and Gehr, 2002) from the market. To avoid costly failures in drug development, pharmacokinetic DDI from CYP450 inhibition is now being investigated earlier in the drug discovery process across the pharmaceutical industry.

Most adverse pharmacokinetic DDIs are attributable to reversible inhibition of CYP450 isozymes. The potential for a discovery compound to be subjected to reversible inhibition is typically investigated by *in vitro* studies to determine IC₅₀ values using recombinant human CYP450 isozymes (Crespi et al., 1997) or pooled human liver microsomes (HLM) (Dierks et al., 2001). That approach weeds out new chemical entities that are potent inhibitors. The IC₅₀ values of new chemical entities from the same chemical series can be used to establish structure-inhibition relationships. Those data, together with a computational model of a specific CYP450 isozyme, can be used to design out the inhibition liability.

Adverse pharmacokinetic DDI can also result from mechanism-based inactivation of CYP450 isozymes, illustrated by some fatal adverse events observed with mibefradil (Posicor) (Krayenbuhl et al., 1999), a potent mechanism-based inactivator (MBI) of CYP3A4 (Prueksaritanont et al., 1999). Irreversible inactivation generally involves metabolism of the inactivator to a reactive metabolite, which covalently modifies the CYP450 isozyme and results in loss of activity (Silverman, 1996). Enzymatic activity can only be restored through *de novo* protein synthesis, therefore inhibitions are prolonged *in vivo* even after elimination of the inactivator. Hence, failure to take into consideration mechanism-based

DMD #3475R

inactivation of CYP450 isozymes can lead to gross underestimation of the potential extent of pharmacokinetic DDI (Bjornsson et al., 2003). Mechanism-based inactivation of CYP450 has also been associated with idiosyncratic toxicity (Tucker et al., 2001), a consequence that is unpredictable because we cannot identify individuals at risk. Therefore, our strategy is to design out structural features responsible for mechanism-based inactivation in drug discovery using a method of screening described herein.

Traditionally, screening for mechanism-based inactivation of a CYP450 isozyme relies on detection of changes in enzymatic activity with and without preincubation of the test compound for a defined period of time. A shift of IC_{50} to a lower value with preincubation indicates mechanism-based inactivation (Favreau et al., 1999); conversely, a higher IC_{50} with preincubation indicates that the test compound is a substrate of the enzyme (Favreau et al., 1999). Measurement of IC_{50} shift is often conducted by incubating a recombinant human CYP isozyme with a substrate that generates a fluorescent metabolite for high throughput screening. However, fluorescent assays are susceptible to interference by fluorescent test compounds or their metabolites. Furthermore, the effect of metabolites generated by one isozyme on other isozymes cannot be tested in a recombinant single-enzyme system. This difficulty is best illustrated by amiodarone, which is an MBI of CYP3A4 but its N-deethylated metabolite inactivates CYP1A2, CYP2B6 and CYP2D6 as well (Ohyama et al., 2000). Therefore, a definitive assay must include pooled HLM that contain a complete array of CYP450 isozymes.

This report describes the development, validation and automation of a method for initial profiling of new chemical entities for mechanism-based inactivation of CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP1A2. This method is based on measuring apparent partition ratios incorporating pooled mixed gender HLM, FDA-recommended marker substrates, and fast analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Potent positive MBIs from the apparent partition ratio (APR) screen are confirmed for time- and concentration-dependent CYP450 inactivations. Reversibility of inactivation

DMD #3475R

is then evaluated by oxidation with potassium ferricyanide and/or dialysis. Application of this strategy has led to our identification of troglitazone, rosiglitazone, and pioglitazone as MBIs of CYP3A4.

Experimental Procedures

Materials. 1'-Hydroxymidazolam, 4-hydroxymidazolam, 6 β -hydroxytestosterone, (\pm)-bufuralol hydrochloride salt, (\pm)-1'-hydroxybufuralol maleate salt, 4'-hydroxydiclofenac, S-(+)-mephenytoin, (\pm)-4'-hydroxymephenytoin, 6',7'-dihydroxybergamottin and bergamottin were purchased from Ultrafine (Manchester, UK). Midazolam maleate salt, diclofenac sodium salt, dextromethorphan hydrobromide monohydrate salt, testosterone, quinidine, troleandomycin, sulfaphenazole, furafylline, α -naphthoflavone, ticlopidine, 17 α -ethynylestradiol, mifepristone, glucose-6-phosphate, NADP⁺, glucose-6-phosphate dehydrogenase, potassium ferricyanide, raloxifene hydrochloride, diltiazem hydrochloride, nifedipine, (\pm)-verapamil hydrochloride, resveratrol, isoniazid, suprofen, tacrine, and carbamazepine were obtained from Sigma (St. Louis, MO). 7-Ethoxyresorufin, resorufin sodium salt, ketoconazole were from ICN Biomedicals (Aurora, OH). Paroxetine, troglitazone, rosiglitazone maleate, and pioglitazone hydrochloride were from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). 1-[(2-Ethyl-4-methyl-1H-imidazol-5-yl)methyl]-4-[4-(trifluoromethyl)-2-pyridinyl]piperazine (EMTPP) was from Ryan Scientific, Inc. (Mt. Pleasant, SC). Tienilic acid and mibefradil were obtained from the compound room of aventis (Bridgewater, NJ). Pooled mixed-gender HLM were from Xenotech LLC (Lenexa, Kansas). Azamulin, S-(+)-N-3-benzyl-nirvanol and supersomes were from BD Biosciences (Bedford, MA). Single 96-well DispoDIALYZER (10,000 MWCO) was purchased from Harvard Apparatus (Holliston, MA). Reagents and materials for Western immunoblotting were from invitrogen (Carlsbad, CA). Aquasil C18 columns and other chromatographic supplies were from Thermo Electron Corporation (Bellefonte, PA). Fisher Scientific International, Inc. (Fairlawn, NJ) was the supplier of other reagent grade chemicals, HPLC grade solvents, and 96-well plates.

DMD #3475R

Characterization of Microsomes. The K_m of each CYP450 isozyme for each batch of microsomes was characterized as published elsewhere (Dierks et al, 2001). The content of individual CYP450 isozymes in microsomes was quantified by densitometry after separation by SDS-PAGE, and identified on Western immunoblots using standard protocols recommended by the vendors.

APR Screens. Incubations in the presence or absence of an NADPH-regenerating system were conducted in 2-mL 96-well plates containing individual test compounds and a known MBI as a positive control. The primary incubations, in total volumes of 200 μ L per well, consisted of 2 mg/mL HLM, 0-50 μ M test compound, 10 mM $MgCl_2$, 2 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate dehydrogenase. The organic solvent was typically DMSO unless otherwise indicated, at a final concentration of 0.5% (v/v). The reactions were initiated by addition of the NADPH-regenerating system and incubated for 1 hr at 37°C to ensure complete inactivation. Residual catalytic activities were assayed by transferring 10- μ L aliquots (20 μ L for CYP1A2) to preincubated secondary assay plates, which were identical to primary plates except that they contained a saturated concentration (5 x K_m) of marker substrate (Table 1) instead of test compound.

Incubations were allowed to proceed for another 20 min and then quenched with 100 μ L of acetonitrile containing 2.5 pg/ μ L dextromethorphan as an internal standard. Microsomal proteins were pelleted by centrifugation at 1,968g for 20 min prior to transfer of the supernatants to new 2-mL 96-well plates for LC/MS/MS analysis. Samples were pooled for analysis of testosterone 6 β -hydroxylase and midazolam 1'-hydroxylase activities. Mean values from duplicate analyses were used to calculate the percent activity remaining, based on values from respective control incubations with solvent alone as 100%. The APRs were then calculated by plotting percent activity remaining as a function of the molar ratio of test compound to CYP450 isozyme (Silverman, 1996).

DMD #3475R

Automation of APR Screen. The APR screen was automated using a Genesis Workstation 200 (Tecan, research triangle park, NC) retrofitted with a Te-MO 96-multichannel pipette head. Automation was validated using mifepristone for CYP3A4 isozyme, with testosterone as the marker substrate. The validation run was conducted using three separate 96-well plates of primary incubation, to simulate screening of five compounds and one positive control for each CYP450 isozyme analyzed. The throughput was six compounds per CYP450 isozyme, in duplicate, per day. LC/MS/MS analysis, data processing and calculation required an additional day.

Time- and Concentration-Dependent Inactivation Assays. All incubations were performed in triplicate in 2-mL 96-well plates with appropriate MBIs as positive controls. The conditions and procedures for the primary and secondary incubations were as described for the screening procedure described above. Concentration ranges for compounds tested in the primary incubation were established from the results of the APR screen. Typically, the selected concentrations corresponded to the molar ratio of test compound to CYP450 isozyme that resulted in inactivation greater than 30%. Residual catalytic CYP450 isozyme activities were assayed at 0, 7.5, 15 and 30 min. Mean values from the triplicate analyses were used to calculate the percent activity remaining, using as 100% the respective control incubation with solvent at time 0.

Reversibility of Inactivation. The reversibility of inactivation of CYP450 isozyme was investigated by oxidation with potassium ferricyanide and also by dialysis, at single concentrations (n = 8) that resulted in more than 50% inactivation. Troleandomycin and mifepristone were included as positive and negative controls respectively; an appropriate quasi-irreversible inhibitor and an MBI for whichever CYP450 isozyme is under investigation may replace them.

For oxidation with potassium ferricyanide, the primary incubates were diluted 2-fold with 50 μ L of either 2 mM potassium ferricyanide or 0.1 M potassium phosphate buffer (pH 7.4). Each mixture was

DMD #3475R

incubated for 15 mins at 37°C, prior to another 20-fold dilution (10-fold for CYP1A2) for a secondary incubation designed to assess residual catalytic CYP450 isozyme activities.

For dialysis, the primary incubates were diluted 10-fold by transferring 20- μ L aliquots to 180 μ L of 0.1 M potassium phosphate buffer (pH 7.4) in each well of a 96-well DispoDIALYZER. Dialysis was conducted with 4 L of 0.1 M potassium phosphate buffer (pH 7.4). The buffer was changed after three hours and dialysis was continued for an additional twenty-one hours. Catalytic CYP450 isozyme activities remaining after dialysis were assayed after 2-fold dilution by transferring 100- μ L aliquots to secondary incubations. The protein concentration in each dialysis well was determined in a 96-well plate format using a BCATM protein assay kit from Pierce Biotechnology, Inc., (Rockford, IL). The primary and secondary incubations were conducted as described in the APR screen. Both reversibility experiments were conducted on the same day, and aliquots of primary incubates were removed for assessment of residual CYP450 isozyme activities without either oxidation by potassium ferricyanide or by dialysis.

Mean values were used to calculate the percent activity remaining, using the solvent control as 100% activity. The reversibility of CYP450 isozyme inactivation was determined by comparing the enzyme activity of samples before and after oxidation by potassium ferricyanide or dialysis. The enzyme activities of dialyzed samples were normalized to protein concentrations.

LC/MS/MS Analysis. All samples were analyzed by LC/MS/MS in the positive-ion electrospray ionization mode. The LC/MS system consisted of a Gilson 306 HPLC equipped with a Gilson 215 autosampler coupled to a Sciex API III⁺ (Applied BioSystems, Foster City, CA), or a Surveyor HPLC and an autosampler coupled to a Quantum AM (Thermo Electron Corporation, San Jose, CA).

All chromatographic separations were carried out on an Aquasil C18 column (50 x 2 mm ID, 5 μ) thermostated at 50°C, using a linear 1.35 min gradient from 95:5 to 5:95 (water:acetonitrile with

DMD #3475R

0.05% formic acid) for analysis of samples from all marker substrates, except for testosterone and midazolam where the starting gradient consisted of 19% acetonitrile. The initial organic content was adjusted for optimum separation of multiple hydroxy metabolites for each marker substrate. A 20- μ L aliquot of sample was typically injected into the column and eluted using a programmed flow rate from 1 to 1.5 mL/min over a 1.35-minute gradient. The total cycle time for each analysis was three minutes.

The LC eluant was split to about 250 μ L/min before being sprayed into the mass spectrometer at 4.5 kV. Nebulization of the LC eluant on the API III⁺ was achieved using nebulizer and auxiliary gases (nitrogen) set at 1.1 L/min and 8000 cc/min, respectively. The sheath and auxiliary gases were set at 35 and 10 arbitrary units, respectively, on the Quantum AM. Desolvation of the solvent droplets on the API III⁺ was aided by heating the nebulizer to 500°C and the interface to 60°C, with a declustering potential of 35V and curtain gas set at 1.8 L/min. Desolvation on the Quantum AM relied on a heated capillary set at 350°C.

Metabolites and internal standard were detected by multiple-reaction monitoring of precursor-product ion pairs (Table 1) at a speed of 2 scans/sec with Q2 settling to reduce cross-talk for API III⁺, or 0.1 sec/scan on the Quantum AM. Precursor ions were selected using Q1 at a resolution of 1.0 Da at half-height for collision-induced dissociation in Q2, and the product ions were mass analyzed with Q3 at unit resolution. The collision gas (argon) was set at 250×10^{13} atoms/cm² for the API III⁺ or 1.5 mTorr on the Quantum AM. The metabolite and the internal standard were monitored using the same collision energy on the API III⁺; optimum collision energy for each ion pair was used on the Quantum AM. Product ions were detected with the electron multiplier set at saturation (4100V) on the API III⁺ or 888V with a gain of 2.7×10^5 on the Quantum AM. Data acquisition and reduction for the API III⁺ were achieved using RAD 2.6 and MacQuan 1.3 while Xcalibur 1.3 was used on the Quantum AM.

Data analysis. Data corresponding to percent activity remaining in the presence or absence of a NADPH-regenerating system were plotted as a function of the molar ratio of test compound to CYP450

DMD #3475R

isozyme, using Sigma Plot (SPSS Science Inc. Chicago, IL). Curvefitting was carried out using single exponential decay with r^2 of at least 0.9. The APR was calculated by a linear regression analysis of the linear-decay portion of the curve and the intercept, with the x -axis corresponding to the APR + 1 (Silverman, 1996). The molar ratio of test compound to CYP450 isozyme that resulted in maximum loss of activity was the lowest molar ratio on the linear regression line that corresponded to the saturated concentrations of test compound.

Apparent inactivation kinetic constants were determined as previously described (Kitz and Wilson, 1962). Briefly, the natural logarithm of percent activity remaining was plotted against the preincubation time for each concentration of test compound investigated. The slope from the linear regression analysis gave the observed inactivation rate constant (k_{obs}) for each concentration. A double-reciprocal plot (also known as the Kitz-Wilson plot) of k_{obs} (y -axis) and the inactivator concentrations (x -axis) produced the apparent k_{inact} (min^{-1}) from the reciprocal of the y -axis intercept and K_I (μM) from the negative reciprocal of the x -axis intercept.

Results

Mechanism-Based Inactivation by APR Screen. APRs were obtained for 19 known MBIs (Table 2). The most potent MBIs detected by this screen were ritonavir, mibefradil and azamulin for CYP3A4, with APRs ranging from 1 to 2 depending on the marker substrate. Other potent compounds (APRs less than 50) inactivated CYP3A4 (raloxifene, bergamottin and 6',7"-dihydroxybergamottin), CYP2C9 (tienilic acid), CYP2D6 (paroxetine) and CYP1A2 (furafylline). Competitive inhibitors and substrates of each of the CYP450 isozymes consistently tested negative by this screen, showing little or no separation of the plus- and minus-NADPH titration curves (data not shown).

Figures 1A through 1L depict titration curves for five MBIs and five competitive inhibitors of CYP450 isozymes in the presence and absence of NADPH. Separation of the plus- and minus-NADPH

DMD #3475R

titration curves occurred for known MBIs like mifepristone (CYP3A4), tienilic acid (CYP2C9), ticlopidine (CYP2C19), paroxetine (CYP2D6) and furafylline (CYP1A2). Plus-NADPH titration curves were associated with residual functional isozyme activity after oxidative metabolism. Minus-NADPH titration curves represented competitive inhibition by residual test compounds. MBIs showed lower residual functional activities in the plus-NADPH titration curves than in the minus-NADPH titration curves. Conversely, there was little or no separation of the plus- and minus- NADPH titration curves for competitive inhibitors like ketoconazole (CYP3A4), sulfaphenazole (CYP2C9), S-(+)-N-3-benzyl-nirvanol (CYP2C19), quinidine (CYP2D6) and α -naphthoflavone (CYP1A2). Any separation of the plus- and minus-NADPH titration curves observed for competitive inhibitors was usually associated with the plus-NADPH titration curves being only slightly higher than those without NADPH.

Figures 1A through 1L also provide information on the APR, the residual functional activity, the maximum loss of activity, and molar ratio at which maximum loss of activity occurred. For example, the APR (residual functional activity) obtained for mifepristone, using testosterone 6 β -hydroxylation and midazolam 1'-hydroxylation, was 5.7 (6%) and 7.2 (14%), respectively. The maximum losses of CYP3A4 activity were 94 and 86% as measured by testosterone 6 β -hydroxylation and midazolam 1'-hydroxylation, respectively, at a preincubation concentration of 10 μ M mifepristone (mifepristone/CYP3A4 ratio of 38). This loss of CYP3A4 activity was comparable to that previously reported using a reconstituted system (He et al., 1999). Losses of CYP3A4 activity were due to inactivation as a result of the higher residual CYP3A4 activity observed at each concentration in the absence of NADPH (open circles in Figures 1A and 1C). Competitive inhibition contributed to about 11 and 30% of CYP3A4 activity measured by testosterone 6 β -hydroxylation and midazolam 1'-hydroxylation, respectively, at the highest preincubation concentration of 50 μ M. This corresponds to a residual concentration of 2.5 μ M, assuming no metabolism. The APRs obtained for ticlopidine, tienilic acid, paroxetine and furafylline were 70.3, 13.2, 6.6 and 28.5 respectively. The maximum losses of

DMD #3475R

enzyme activity for ticlopidine, tienilic acid, paroxetine and furafylline were 85, 89, 73, and 72% at molar ratios of compound to CYP450 isozyme of 156, 61, 16, and 142, respectively.

The APR screen also detected quasi-irreversible inhibitors, including troleandomycin, diltiazem, nifedipine and verapamil (Table 2), which formed a nitrosoalkane metabolite-intermediate (MI) complex with the enzyme. Troleandomycin was the most potent quasi-irreversible inhibitor investigated, with APRs of 4.5 and 4.0 using testosterone and midazolam respectively, as marker substrates.

Accuracy and Precision of the APR Screen. Data concerning intra- and inter-day precision and accuracy for determination of each MBI by the APR screen are presented in Table 3. Intra-day precision ranged from 6.7 to 20.3 % coefficient of variation (CV) for MBIs of the five major CYP450 isozymes; inter-day CVs ranged from 1.8 to 22.4 %. Measured APRs of 12.6, 78.1 and 35.3 for tienilic acid, ticlopidine, and furafylline were respectively 1.1-, 3.0- and 5.6-fold greater than previously reported values (tienilic acid: CYP2C9, $r = 12.0$, Lopez-Garcia et al., 1994; ticlopidine: CYP2C19, $r = 26.0$, Ha-Duong et al., 2001; furafylline: CYP1A2, $r = 5.6$, Kunz and Trager, 1993).

Automation. The precision obtained for the automated APR screen using 96-multi channels Te-MO was 7.8 %CV. The APR for mifepristone from the automated screen was 6.4 ± 0.5 compared to an APR of 6.0 ± 0.4 obtained by the manual method.

Time- and Concentration-Dependent Inactivation. Kitz-Wilson plots in Figure SII (Supporting Information) showed that inactivations of CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP1A2 by mifepristone, tienilic acid, ticlopidine, paroxetine and furafylline were dependent on time and concentration, as indicated by a linear relationship with an r^2 of at least 0.9. Competitive inhibitors did not display time- and concentration-dependent CYP450 isozyme inactivation (data not shown).

Intra-day precision for k_{inact} ranged from 3.1 to 31.4 and K_I from 8.4 to 44.4 %CV (Table SII, Supporting Information). Inter-day precision was slightly higher for k_{inact} (31.0 to 57.2 %CV) but the precision from inter-day determination of K_I (7.2 to 45.3 %CV) was comparable to intra-day. The intra-

DMD #3475R

day precision obtained for k_{inact} and K_I of each MBI was comparable to the corresponding precision values reported elsewhere for CYP2C9 (Lopez-Garcia et al., 1994), CYP2C19 (Ha-Duong et al., 2001), CYP2D6 (Bertelsen et al., 2003) and CYP1A2 (Clarke et al., 1994). The differences between our observed mean values of k_{inact} and K_I and those previously reported ranged from -4.5 to $+23.7$ and -16.0 to $+2.0$ -fold.

Reversibility of CYP450 Isozyme Inactivation. Data from assessment of reversibility of CYP450 isozyme inactivation by oxidation with potassium ferricyanide and by dialysis are tabulated in Table SI2 (Supporting Information). Only CYP3A4, when inactivated by either troleandomycin or verapamil, was reversible by oxidation with potassium ferricyanide. However, neither CYP2D6 inactivated by paroxetine nor CYP3A4 inactivated by mifepristone or mibefradil were reversible by oxidation with potassium ferricyanide. The intra- and inter-day reproducibility of the experiments for assessing reversibility of CYP450 isozyme inactivation by oxidation with potassium ferricyanide had a CV of <11 %.

Activity of CYP3A4 inactivated by azamulin, mibefradil or mifepristone was not completely recovered by dialysis. The irreversibility of azamulin-inactivated CYP3A4 was consistent with mechanism-based inactivation as previously reported (Stresser et al., 2003). However, the activity of CYP3A4 inactivated by troleandomycin, verapamil or nicardipine was completely restored by dialysis. Interestingly, only about 40% of bergamottin-inactivated CYP3A4 activity was restored by dialysis, and partial reversibility after dialysis was also observed for paroxetine-inactivated CYP2D6, as indicated by restoration of about 42 and 21% activity of enzyme that had been inactivated by 0.625- and 20- μ M paroxetine, respectively. The intra- and inter-day precision of dialysis in 96-well format was <12 %CV. Agreement of the current data with those previously reported suggested that dialysis in 96-well plates was just as effective as using single dialysis cartridges. In addition to positive and negative controls,

DMD #3475R

one can use restoration of enzyme activity in samples lacking NADPH to check for effectiveness of dialysis.

Screening of Unknowns. Data from profiling troglitazone, rosiglitazone and pioglitazone for mechanism-based inactivation of CYP3A4 by the APR screen are presented in Figure 2. The separation of the titration curves of each compound, with and without NADPH, using either of two marker substrates of CYP3A4 suggested that the inactivation of CYP3A4 by troglitazone, rosiglitazone and pioglitazone was mechanism-based, with APRs (residual functional isozyme activity) of 45.7 (4%, testosterone) and 51.0 (5%, midazolam) for troglitazone, 185.2 (12%, testosterone) and 226.5 (21%, midazolam) for rosiglitazone, and 277.3 (28%, testosterone) and 407.5 (36%, midazolam) for pioglitazone. Hence, troglitazone was 4.1-fold (testosterone) and 4.4-fold (midazolam) more potent than rosiglitazone, and 6.1-fold (testosterone) and 8.0-fold (midazolam) more potent than pioglitazone. Rosiglitazone was 1.5-fold (testosterone) and 1.8-fold (midazolam) more potent than pioglitazone.

Time- and concentration-dependency of inactivation of CYP3A4 by troglitazone, rosiglitazone and pioglitazone was confirmed by the Kitz-Wilson plots shown in Figure 3. The inactivation kinetic constants were $k_{inact} = 0.0335 \text{ min}^{-1}$ and $K_I = 5.0 \text{ }\mu\text{M}$ for troglitazone, $k_{inact} = 0.0195 \text{ min}^{-1}$ and $K_I = 11.9 \text{ }\mu\text{M}$ for rosiglitazone, and $k_{inact} = 0.0112 \text{ min}^{-1}$ and $K_I = 10.4 \text{ }\mu\text{M}$ for pioglitazone. Inactivation clearance data indicated that troglitazone was 4.2-fold more potent than rosiglitazone and 6.1-fold more potent than pioglitazone; rosiglitazone was 1.5-fold more potent than pioglitazone. Overnight dialysis did not restore the activity of CYP3A4 inactivated by these compounds (Table 4), evidence that inactivation of CYP3A4 by any of the three glitazones was irreversible.

Discussion

Development and Validation of an APR Screen. The titration method commonly used to determine partition ratios (Silverman, 1996) was recently applied to human CYP450 isozyme (Lin et al.,

DMD #3475R

2002). We report here for the first time the development of an APR screen by a titration method using HLM.

Our APR screen for MBIs was validated by correct identification of 19 known MBIs, with no false-positive results. The detection of MBIs with approximately 7000-fold differences in APRs (Table 2) indicates the wide dynamic range of the screen. This wide range may require retesting of some compounds at different concentration ranges depending on their inactivation potency and, if present, any competitive-inhibition potency as well. This issue is illustrated by our identification of ritonavir as an MBI of CYP3A4 from the APR screen at a concentration only 10-fold lower than the default concentration range (Figure SI2, Supporting Information). The sensitivity of the screen was underscored by the detection of weak MBIs with $k_{inact}/K_I < 1 \text{ min}^{-1}\text{mM}^{-1}$, such as isoniazid for CYP3A4, CYP2C19 and CYP1A2 (Wen et al., 2002) and diclofenac for CYP3A4 (Masubuchi et al., 2002).

Intra- and inter-day precision of <25 %CV achieved for APRs of five major CYP450 isozymes (Table 3) are acceptable for ranking compounds in discovery. We have no explanation for lower intra-day precision for all the isozymes except CYP1A2. Better precision might be obtained by replacing current internal standard for bioanalysis with corresponding stable isotope-labeled metabolite of each marker substrate (Walsky and Obach, 2004). The accuracy of the method is difficult to demonstrate because no APR using HLM has been reported to date. Despite experimental differences, reasonably good agreement is indicated by less than 6-fold differences from reported intrinsic partition ratios of tienilic acid, ticlopidine, furafylline, 17α -ethynylestradiol, EMTPP and suprofen. However, agreement is closer if the intrinsic partition ratios of 17α -ethynylestradiol (Lin et al., 2002), EMTPP (Hutzler et al., 2004) and suprofen (O'Donnell et al., 2003) are estimated from intercepts with the x -axis as originally defined (Silverman, 1996), and applied to other enzymes (Pochet et al., 2000). The reduction in potency after moving from a recombinant to a microsomal system could be explained by a different degree of protein binding, and/or reduction of substrate due to metabolism by other CYP450 isozymes.

DMD #3475R

All CYP3A4 MBIs (except isoniazid) we detected using testosterone and midazolam had the same potency ranking and differences of less than 2-fold in their APRs. Interestingly, the APRs and residual CYP3A4 activities measured using midazolam were consistently higher than the results with testosterone, suggesting that the contribution of midazolam to inactivation is negligible. The small difference in the APRs obtained with either marker substrate suggests that covalent modification of testosterone- and midazolam-binding sites of CYP3A4 by each inactivator is comparable. However, loss of enzyme activity associated with covalent modification is greater for the testosterone-binding site, as indicated by lower % residual CYP3A4 activity (Table 2).

Inactivation Kinetic Constants. To assess the potential magnitude of pharmacokinetic DDI by potent MBIs with APRs less than 50, inactivation kinetic constants must be determined before a compound can be brought into development. The challenge for measuring k_{inact} and K_I is to select a concentration range for the experiment (Silverman, 1996); that choice is made easier by data from the APR screen.

In general, higher variability is associated with this assay, an observation consistent with at least one literature report (Voorman et al., 1998). The precision of the assay might be improved by using a stable isotope-labeled metabolite of each marker substrate because of similarity of mass spectrometric and chromatographic behaviors (Watson, 1997).

Reversibility of CYP Inactivation. In addition to data for time- and concentration-dependent inactivation, reversibility of inactivation must be assessed for confirming any potent inactivator indicated by the APR screen. MBIs carry a risk of idiosyncratic toxicity (Tucker et al., 2001) in addition to the pharmacokinetic DDI liability observed with competitive or quasi-irreversible inhibitors. Hence, an ability to differentiate an MBI from a quasi-irreversible inhibitor will allow better decision-making in nominating a compound for development. The reversibility of the nitrosoalkane-MI P450 complex by either oxidation with potassium ferricyanide (Franklin, 1991; Ma et al., 2000) or dialysis (Ma et al.,

DMD #3475R

2000), suggests that an MBI can be differentiated from this type of quasi-irreversible inhibitor. Hence, we have validated the reversibility of CYP450 isozyme inactivation in our experiments by oxidation with potassium ferricyanide and dialysis, with an acceptable precision of <12 %CV (Table SI2, Supporting Information). Our data indicate that oxidation with potassium ferricyanide and dialysis is equally effective in dissociating nitrosoalkane-MI complexes, consistent with that reported elsewhere for nitrosoalkane-MI complexes (Ma et al., 2000). Alternatively, nitrosoalkane-MI complexes can be detected by analysis of substrate-induced difference spectra (Franklin, 1991).

Chemical structures responsible for quasi-irreversible CYP450 inhibition have included dialkylamino (Bensoussan et al., 1995) and 3,4-methylenedioxyphenyl (Murray, 2000) functionalities. As the dialkylamino functionality is often used to improve the druggable property of a compound, compound with such functionality must be checked for potential to cause quasi-irreversible inhibition if we want to design out this liability. For example, inactivation of CYP3A4 by mifepristone has been associated with formation of nitrosoalkane-MI complexes through oxidation of the dimethylamino moiety (Jang and Benet, 1998) or through covalent binding to apoCYP3A4 by the reactive ketene metabolite produced from oxidation of pro-1-nyl moiety (He et al., 1999). Our data indicate that inactivation of CYP3A4 by mifepristone is not reversible (Table SI2, Supporting Information), confirming the involvement of the prop-1-nyl moiety as previously reported (He et al., 1999). This correct identification of functionality on a compound is necessary for designing out mechanism-based inactivation liability during lead optimization.

3,4-methylenedioxyphenyl compounds, via a carbene-MI complex or through covalent binding by ortho quinone, can inactivate CYP450 isozymes. In our experiments the carbene-MI CYP2D6 complex resulting from paroxetine (Bertelsen et al., 2003) was not reversible by oxidation with potassium ferricyanide, despite a report that the carbene-MI CYP3A4 complex resulting from dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene-dioxybiphenyl-2,2'-dicarboxylate (DDB) was completely

DMD #3475R

reversible (Kim et al., 2001). However, the concentration-dependent partial reversibility of paroxetine-inactivated CYP2D6 by dialysis we have reported here is consistent with that observed for DDP-inactivated CYP3A4 (Kim et al., 2001). Whether inactivation of CYP2D6 activity by 20- μ M paroxetine is reversible by dialysis is unclear, however, because of a comparable gain in activity for the dialyzed sample in the absence of NADPH. Therefore, identification of carbene-MI complex in paroxetine-inactivated CYP2D6 has to rely on substrate-induced difference in spectral analysis (Bertelsen et al., 2003) or on electrospray ionization mass spectrometry, for detection of covalent binding by ortho quinone (Hutzler et al., 2004).

Screening of Unknowns. Compounds presenting a structural alert for reactive metabolites and/or formation of MI complexes are candidates for screening for potential mechanism-based inactivation. For example, the three glitazones (troglitazone, rosiglitazone and pioglitazone) are able to generate reactive metabolites from oxidation of chromane and/or 2,4-thiazolidinedione moieties (Kassahun et al., 2001).

In our experiments, troglitazone was a potent MBI for CYP3A4, with an APR of about 50. Inactivation was dependent on time and concentration, and was not reversible by dialysis. However, it is not clear which functionality on troglitazone is responsible for inactivation. Our data on APRs and inactivation clearance numbers indicate that all three glitazones are MBIs of CYP3A4, and their order of potency for inactivation is troglitazone > rosiglitazone > pioglitazone. Structurally, the three glitazones share a 2,4-thiazolidinedione functionality. Reactive metabolites from bioactivation of 2,4-thiazolidinedione moiety can inactivate CYP3A4. However, troglitazone is the only one containing a chromane moiety; instead, rosiglitazone has a dialkylamino-pyridine and pioglitazone has a dialkylpyridine group. Formation of quinone methide from chromane might contribute to the greater potency of troglitazone for inactivating CYP3A4. The less effective formation of covalent adducts in CYP3A4 by rosiglitazone and pioglitazone, combined with the much lower doses generally prescribed (less than 10

DMD #3475R

mg/day; Utrecht, 2003) may explain the lacking of idiosyncratic hepatotoxicity and pharmacokinetic DDI of those drugs, compared to troglitazone, in clinical settings. This example clearly illustrates that the APR screen has adequate resolution for identifying problematic functionalities for lead optimization.

In conclusion, we have developed and validated an automated screen for determining APRs to detect mechanism-based inactivation of CYP3A4, CYP2C19, CYP2C9, CYP2D6 and CYP1A2. The APR screen has the resolution and sensitivity for detecting MBIs and quasi-irreversible inhibitors. Potent inactivators identified in APR screens are then confirmed by time- and concentration-dependent inactivation experiments. Differentiation of mechanism-based inactivation from quasi-irreversible inhibition involving the nitrosoalkane-MI complex can be achieved by oxidation with potassium ferricyanide or by dialysis. A strategy based on structural alerts for detecting MBI successfully identified troglitazone, rosiglitazone and pioglitazone as CYP3A4 MBIs.

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Supporting Information. Table SII: Intra- and inter-day assay performance calculated from measurements of inactivation kinetic constants (k_{inact} and K_I) of reported MBIs of 5 major CYP450 isozymes. Table SI2: Data (% of control solvent) from validation of reversibility of inactivation of CYP3A4 and CYP2D6 isozymes by oxidation with potassium ferricyanide or dialysis are presented as mean \pm S.D. of 8 determinations. Figure SII: Data from validation of time- and concentration-dependent inactivation assays are presented as Kitz-Wilson plots: A) mifepristone for CYP3A4, B) tienilic acid for CYP2C9, C) ticlopidine for CYP2C19, D) paroxetine for CYP2D6 and E) furafylline for CYP1A2. Each data point is a mean of triplicate analyses and the difference of each reading is $\leq 20\%$ from the mean. The data were analyzed by linear regression analysis with $r^2 \geq 0.9$. Figure SI2: Evaluation of ritonavir for mechanism-based inactivation of CYP3A4 by an APR screen using testosterone (A) and midazolam (B) as marker substrates.

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DMD #3475R

Legends

Figure 1. Profiling for mechanism-based inactivation of 5 CYP450 isozymes by APR screen. Titration curve corresponding to A) mifepristone [testosterone] for CYP3A4, B) ketoconazole [testosterone] for CYP3A4, C) mifepristone [midazolam] for CYP3A4, D) ketoconazole [midazolam] for CYP3A4, E) tienilic acid for CYP2C9, F) sulfaphenazole for CYP2C9, G) ticlopidine for CYP2C19, H) S-(+)-N-3-benzyl-nirvanol for CYP2C19, I) paroxetine for CYP2D6, J) quinidine for CYP2D6, K) furafylline for CYP1A2, and L) α -naphthoflavone for CYP1A2. The closed and opened circles referred to samples with and without NADPH, respectively. Each data point is a mean of duplicate analyses. The data is fitted to an exponential decay function with number of variables determined by best fit based on correlation coefficient (r^2) ≥ 0.9 .

Figure 2. Troglitazone, rosiglitazone and pioglitazone were evaluated for mechanism-based inactivation of CYP3A4 by APR screen using testosterone and midazolam as marker substrates. The titration curves with (closed) and without (opened) NADPH for troglitazone [(A, testosterone), (B, midazolam)], rosiglitazone [(C, testosterone), (D, midazolam)] and pioglitazone [(E, testosterone), (F, midazolam)]. Each data point is a mean of duplicate analyses. The data is fitted to an exponential decay function with number of variables determined by best fit based on $r^2 \geq 0.9$. The chemical structure of troglitazone, rosiglitazone and pioglitazone is depicted in Figure 2 G, H and I, respectively.

Figure 3. Troglitazone, rosiglitazone and pioglitazone were evaluated for time- and concentration-dependent inactivation of CYP3A4. The data are presented as Kitz-Wilson plot for troglitazone (A), rosiglitazone (B) and pioglitazone (C). Each data point is a mean of triplicate analyses and the difference of each reading is $\leq 20\%$ from mean. The data was analyzed by linear regression analysis with $r^2 \geq 0.9$.

Table 1. Selected mechanism-based inactivation assay conditions including marker substrates, concentrations, metabolites, MRM transitions, positive and negative controls.

Isozyme	Marker substrate	Concentration (μM)	Metabolite	MRM	Positive control	Negative control
CYP3A4	Testosterone	200	6β-Hydroxytestosterone	305→269	Mifepristone	Ketoconazole
CYP3A4	Midazolam	29	1'-Hydroxymidazolam	342→324	Mifepristone	Ketoconazole
CYP2C9	Diclofenac	69	4'-Hydroxydiclofenac	312→230	Tienilic acid	Sulfaphenazole
CYP2C19	S-Mephenytoin	274	4'-Hydroxymephenytoin	235→150	Ticlopidine	S-(+)-N-3-Benzyl-nirvanol
CYP2D6	Bufuralol	56.5	1'-Hydroxybufuralol	278→186	Paroxetine	Quinidine
CYP1A2	7-Ethoxyresorufin	10	Resorufin	214→186	Furafylline	α-Naphthoflavone
Internal Standard	Dextromethorphan	2.5 pg/μL		272→171		

Stock solutions of marker substrates were prepared in ethanol

Table 2. Application of the APR screen to detect MBIs using reported MBIs and quasi-irreversible inhibitors. Data is reported as APR (residual functional CYP activity)

Compound	CYP3A4		Measured APR	CYP2C9	CYP2D6	CYP1A2
	Testosterone	Midazolam	CYP2C19			
Ritonavir	1.0 (2%)*	1.4 (2%)*				
Mibefradil	1.4 (3%)	2.0 (11%)				
Azamulin	1.6 (2%)	2.1 (8%)				
Troleandomycin	4.5 (27%)	4.0 (39%)				
Mifepristone	5.5 (6%)	5.8 (17%)				
Raloxifene	7.9 (7%)	11.2 (30%)				
Bergamottin	10.7 (1%)	12.4 (4%)				654.2 (47%)
Diltiazem	11.7 (37%)	28.7 (56%)				
6',7'-Dihydroxybergamottin	13.3 (1%)	16.5 (5%)				
Nicardipine	31.4 (2%)	41.3 (10%)				
Verapamil	39.6 (26%)	96.2 (42%)				
Resveratrol	97.2 (7%)	168.9 (25%)				166.6 (10%) [#]
17 α -ethynylestradiol	260.0 (5%) [#]	277.0 (11%) [#]				
Isoniazid	2148.5 (32%) [#]	5187.0 (64%) [#]	13491.3 (55%) [#]			7254.7 (30%) [#]
Diclofenac	6072.2 (11%) [~]	5652.0 (10%) [~]				
Ticlopidine			88.0 (11%)			
Tienilic acid				11.5 (9%)		
Suprofen				237.7 (53%) [#]		
Paroxetine					5.6 (24%)	
EMTPP					213.5 (28%) ⁺	
Furafylline						28.9 (25%)
Tacrine						1734.0 (47%)
Carbamazepine						4497.5 (63%) [#]

[~]: Concentrations tested from 0 to 2000 μ M

[#]: Concentrations tested from 0 to 500 μ M

⁺: Concentrations tested from 0 to 250 μ M

^{*}: Concentrations tested from 0 to 5 μ M

DMD #3475R

Table 3. Intra- and inter-day assay performance of the APR screen for reported MBIs of 5 CYP450 isozymes. The intra-day is from the average of the APRs from 3 different incubations in duplicate on the same day, whereas the inter-day is from the average of the APRs from 3 different incubations in duplicate on 3 different days.

Analysis	APRs					
	CYP3A4		CYP2C9	CYP2C19	CYP2D6	CYP1A2
	Mifepristone TTS	MDZ	Tienilic acid	Ticlopidine	Paroxetine	Furafylline
Intra-day						
1	5.6	6.7	15.0	80.8	7.4	45.8
2	6.1	7.1	13.2	70.3	6.6	43.9
3	6.4	8.3	13.2	81.0	9.7	53.7
Mean ± SD	6.0 ± 0.4	7.4 ± 0.8	13.8 ± 1.0	77.4 ± 6.1	7.9 ± 1.6	47.8 ± 5.2
Analysis	APRs					
	CYP3A4		CYP2C9	CYP2C19	CYP2D6	CYP1A2
	Mifepristone TTS	MDZ	Tienilic acid	Ticlopidine	Paroxetine	Furafylline
Inter-day						
1	5.6	6.7	13.2	81.0	5.8	33.5
2	5.7	7.4	12.8	79.1	6.0	43.9
3	5.5	6.6	11.8	74.2	5.9	28.5
Mean ± SD	5.6 ± 0.1	6.9 ± 0.4	12.6 ± 0.7	78.1 ± 3.5	5.9 ± 0.1	35.3 ± 7.9

TTS: Testosterone

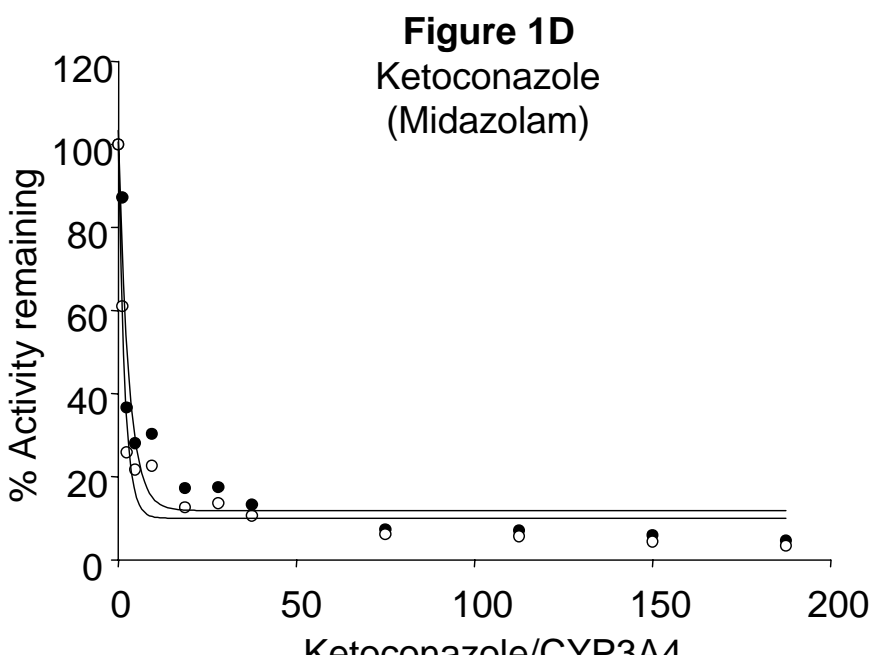
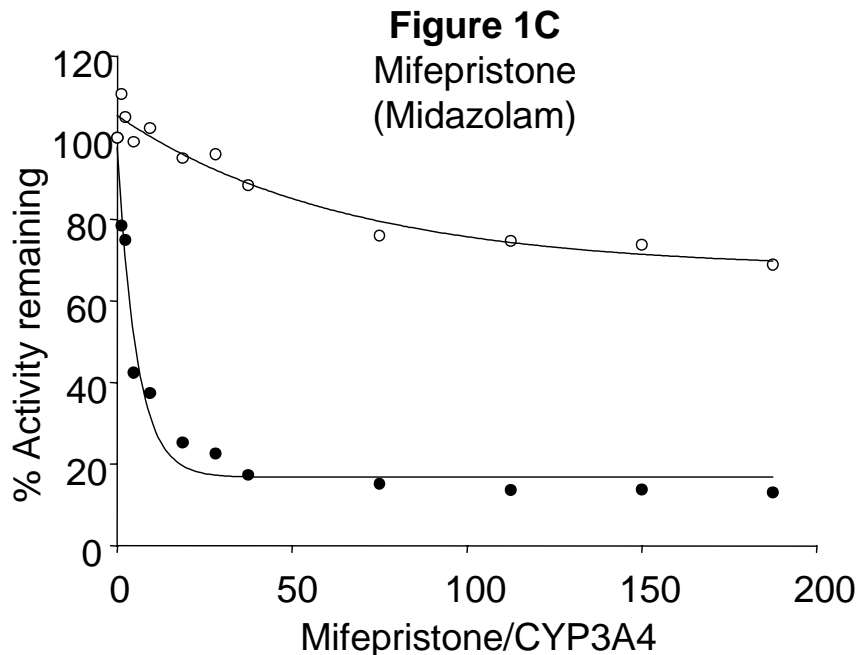
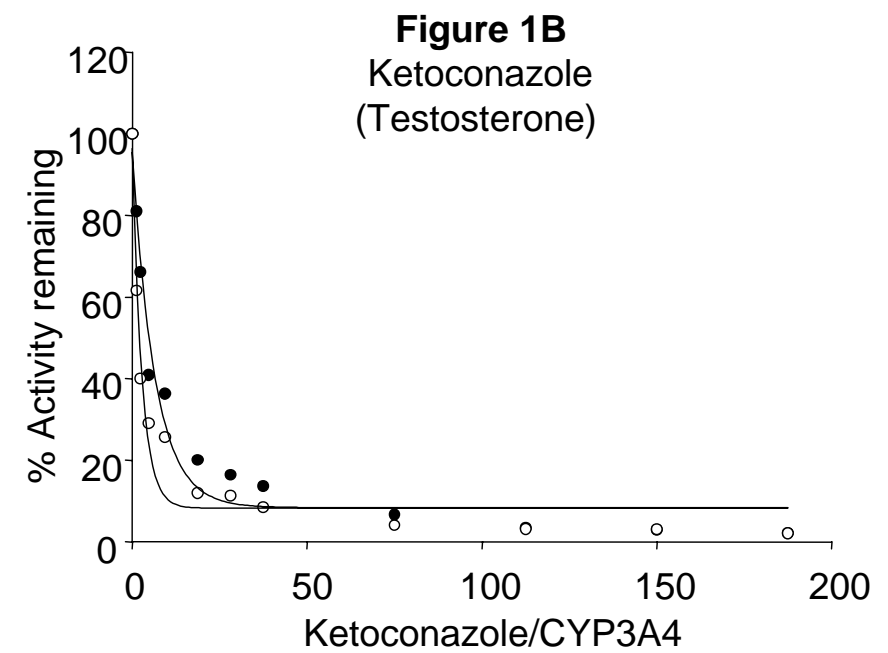
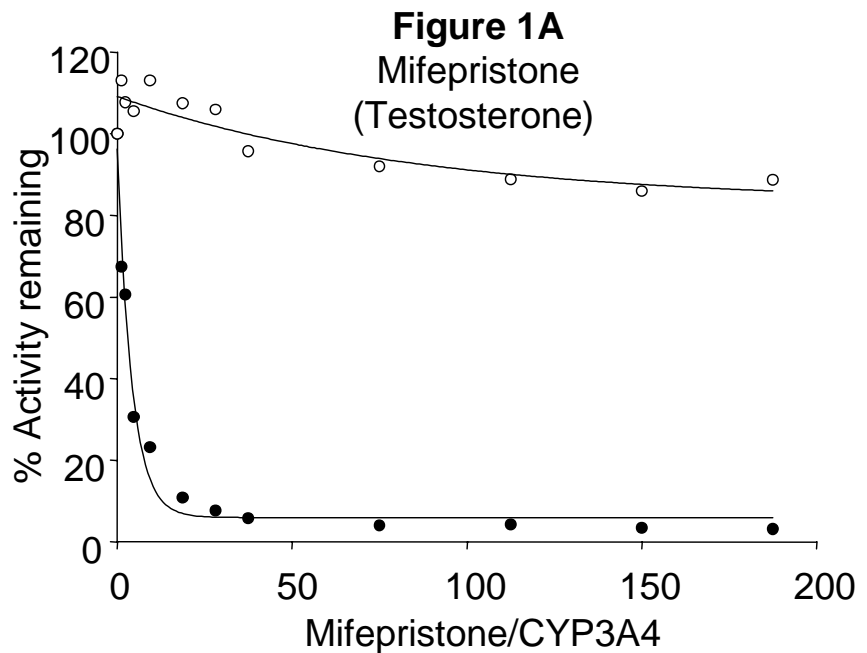
MDZ: Midazolam

DMD #3475R

Table 4. Data (% of solvent control) from evaluation of reversibility of inactivation of CYP3A4 by troglitazone, rosiglitazone and pioglitazone is reported as mean \pm S.D. of 8 determinations.

Compound	Concentration (μ M)	Without dialysis		With dialysis	
		+NADPH	-NADPH	+NADPH	-NADPH
Troglitazone	25	34.2 \pm 3.2	90.0 \pm 4.2	29.2 \pm 1.2	77.0 \pm 5.7
Rosiglitazone	25	59.2 \pm 3.1	106.3 \pm 4.6	57.5 \pm 2.8	91.3 \pm 3.6
Pioglitazone	25	78.8 \pm 4.7	107.2 \pm 6.1	72.5 \pm 4.2	88.1 \pm 4.4*

*Sample without NADPH is significantly different from that with NADPH ($p < 0.01$) by the Student's t test



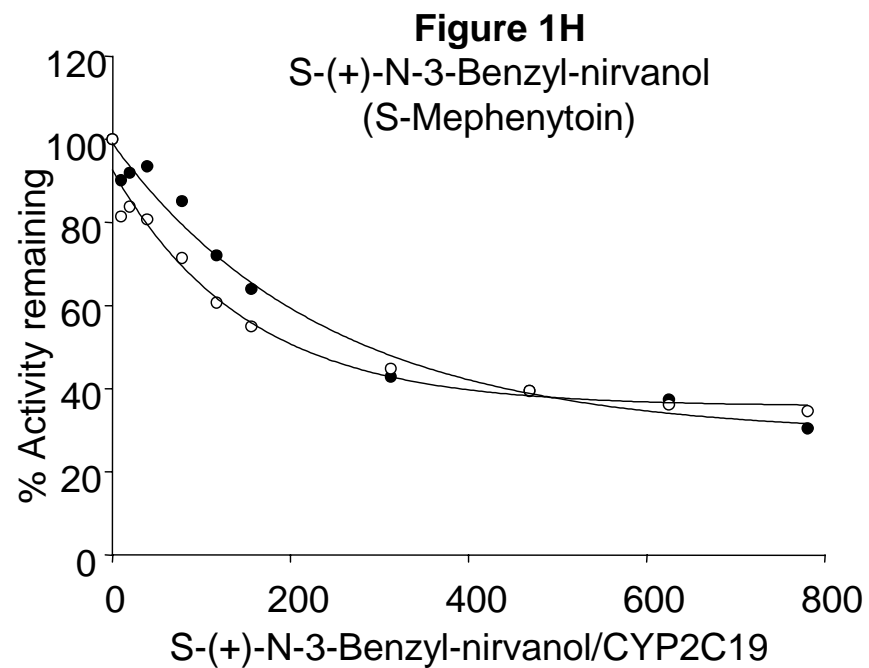
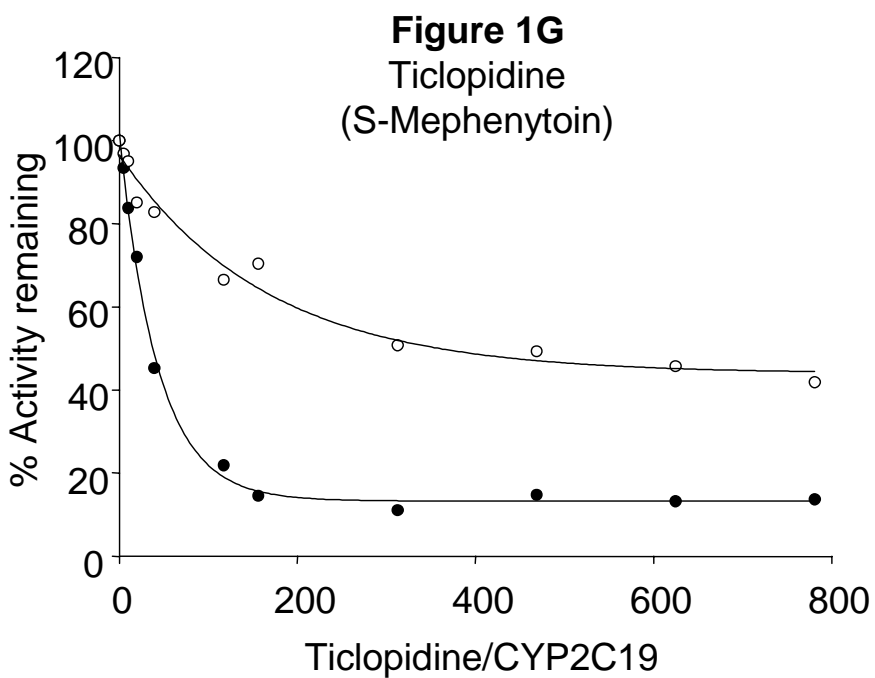
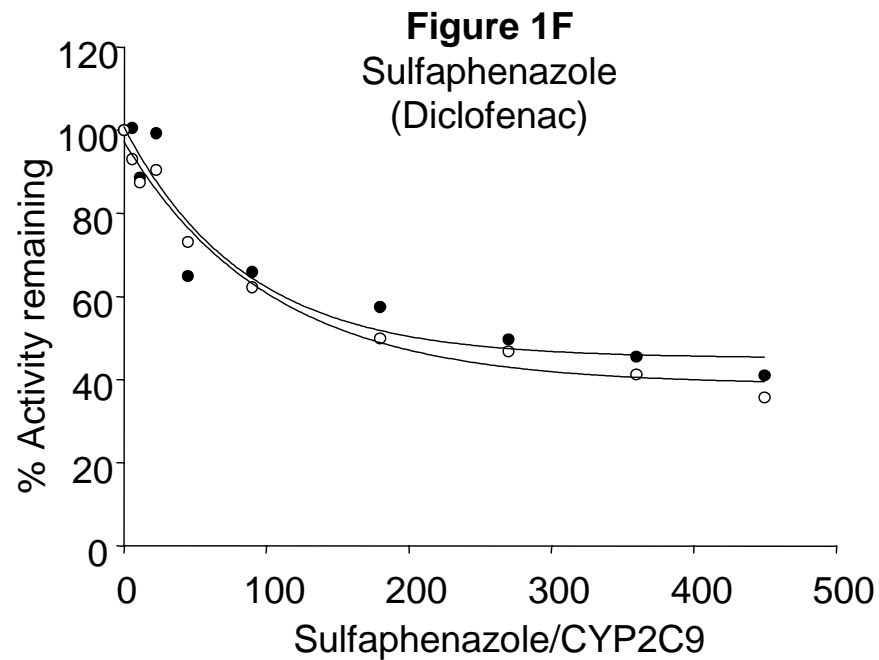
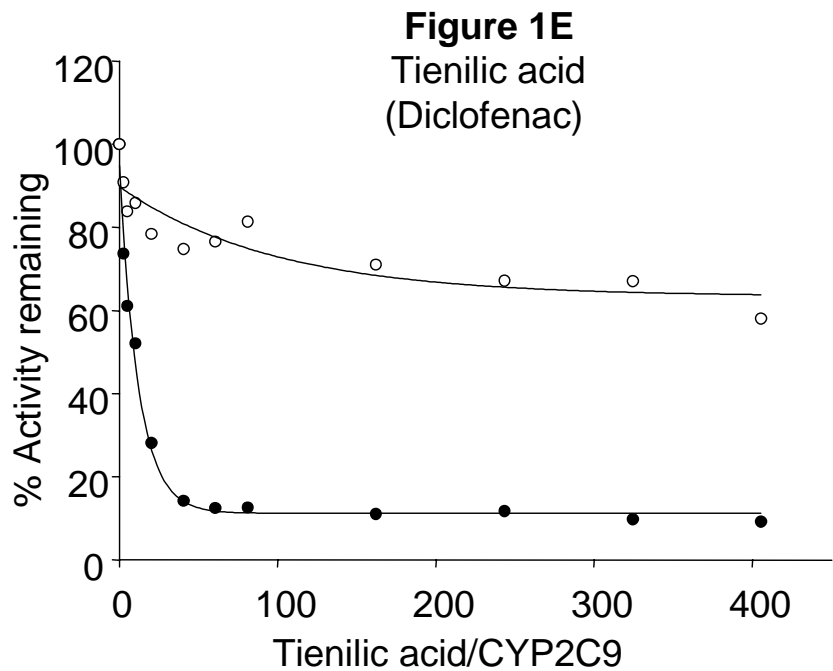


Figure 1

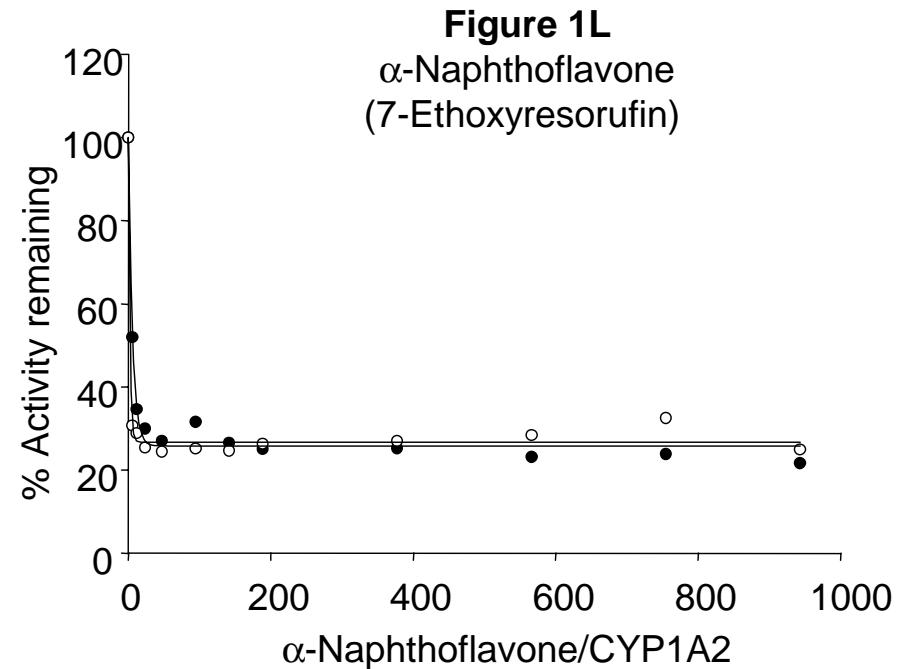
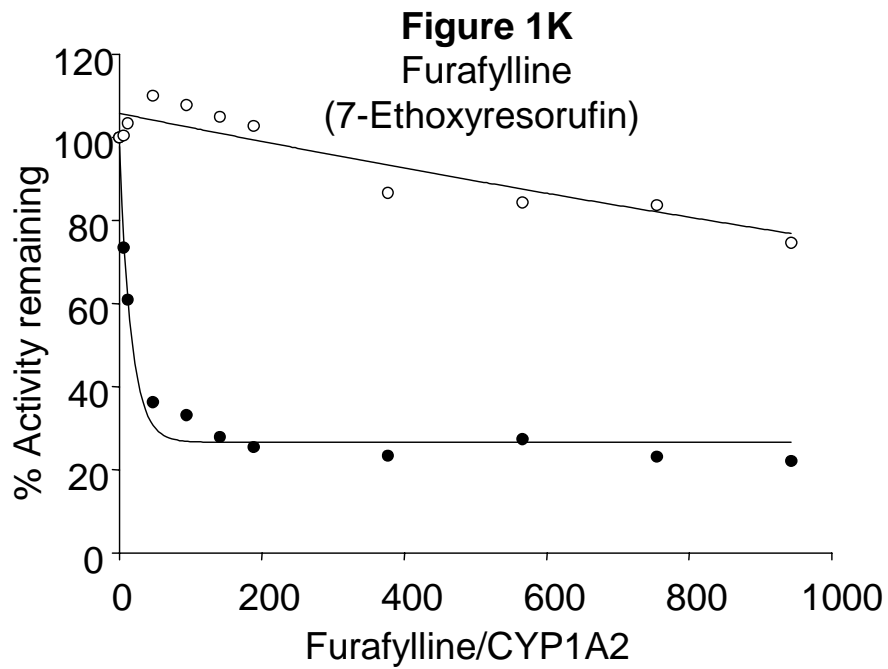
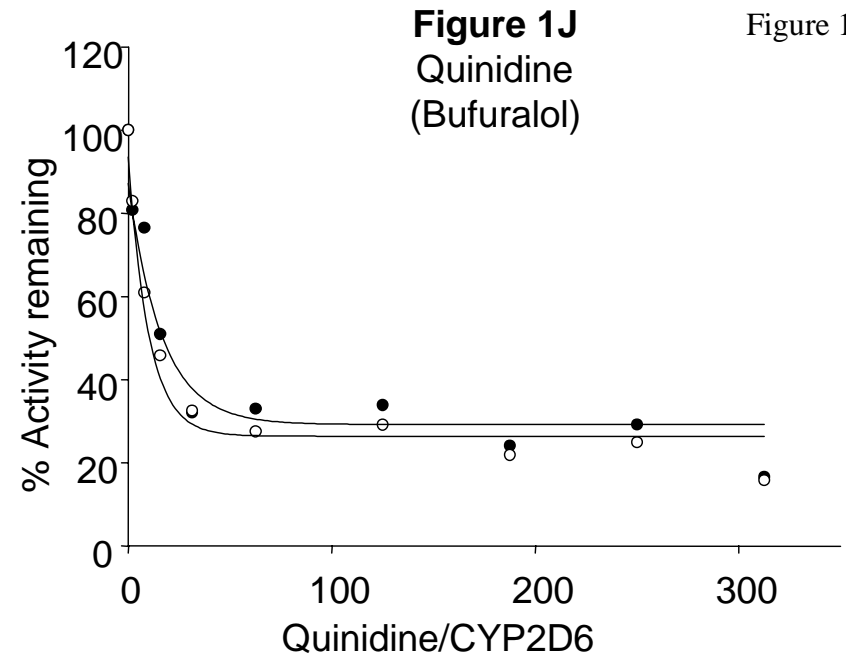
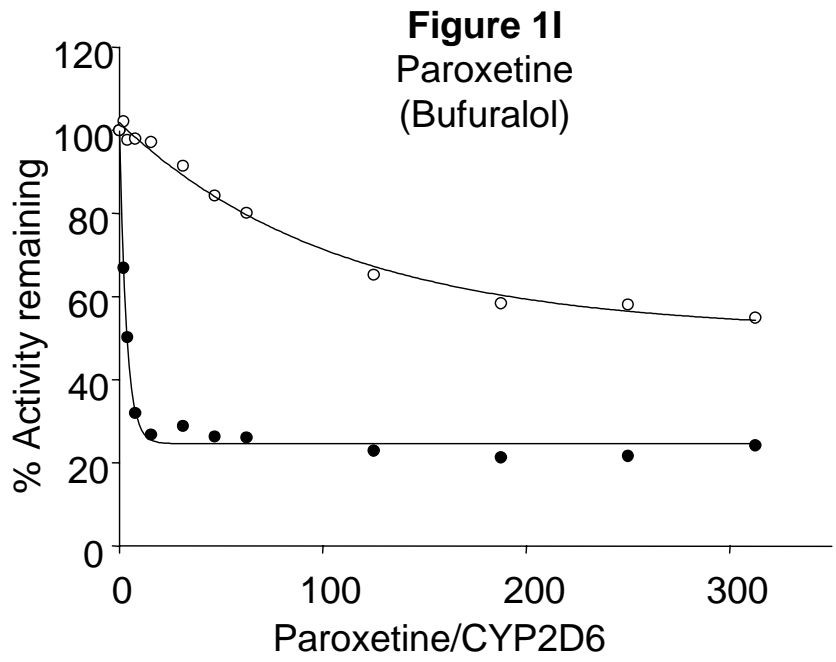


Figure 2A
Troglitazone
(Testosterone)

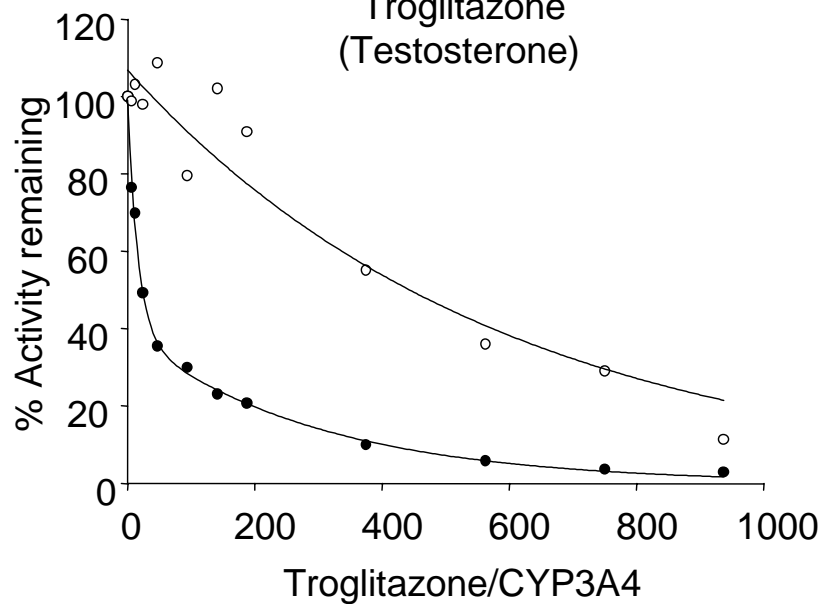


Figure 2B
Troglitazone
(Midazolam)

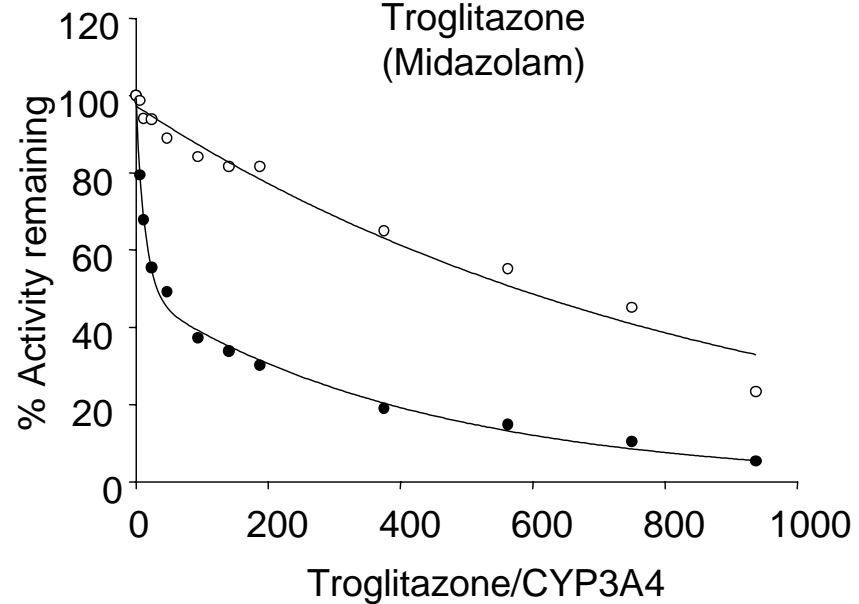


Figure 2C
Rosiglitazone
(Testosterone)

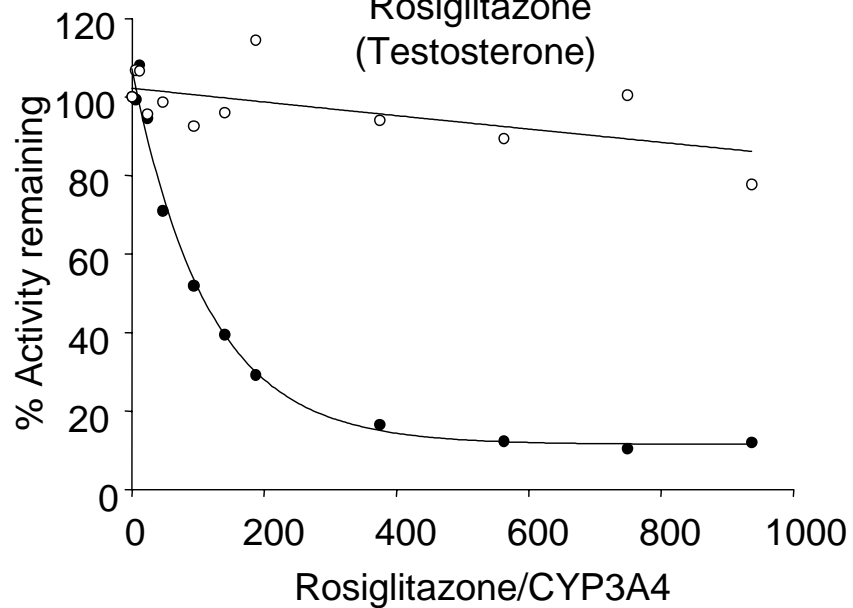
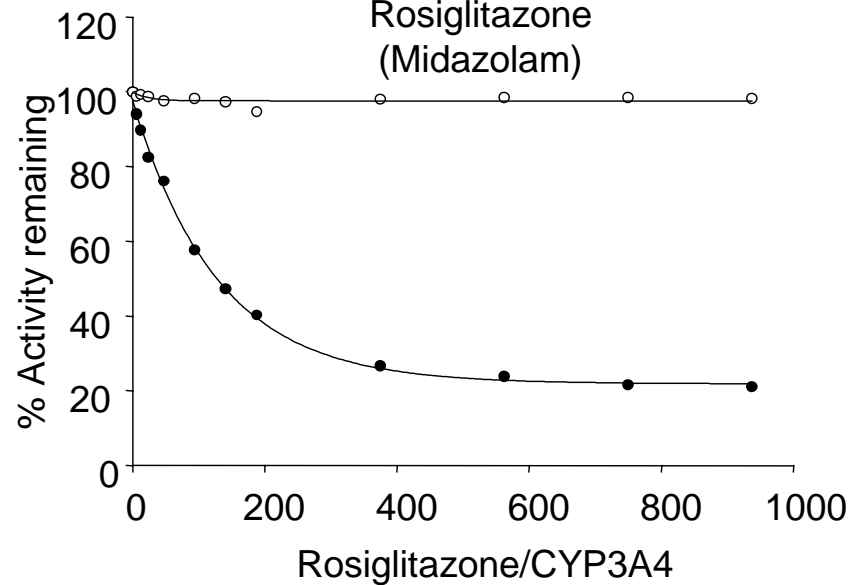


Figure 2D
Rosiglitazone
(Midazolam)



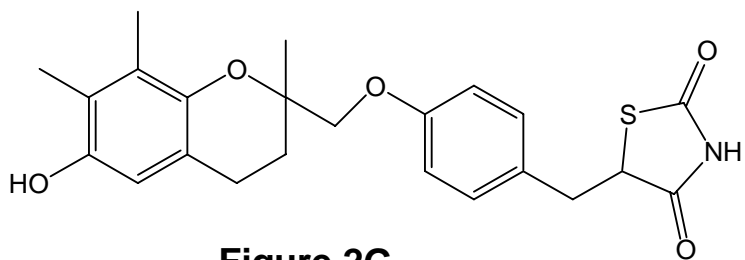
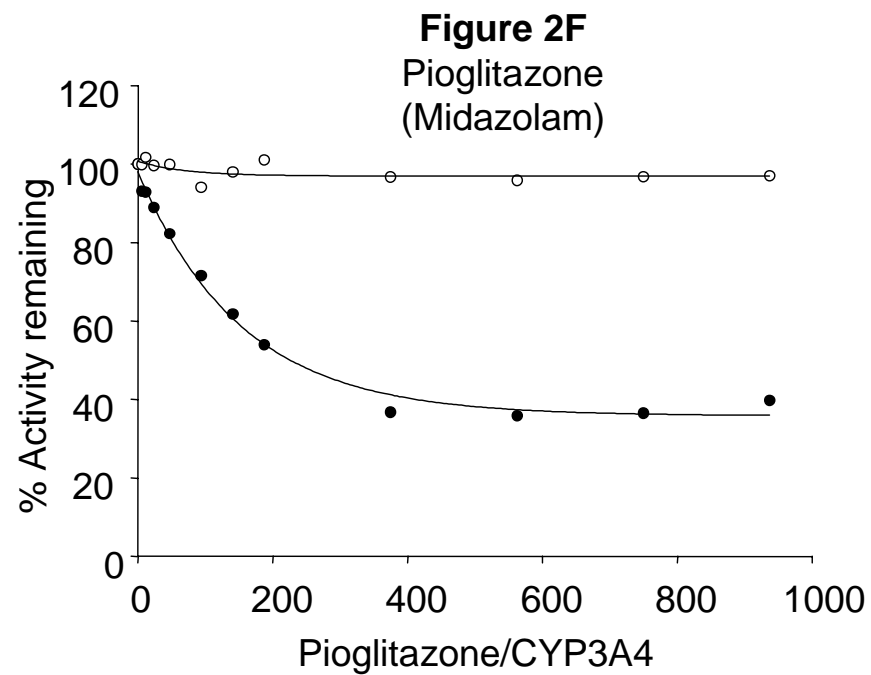
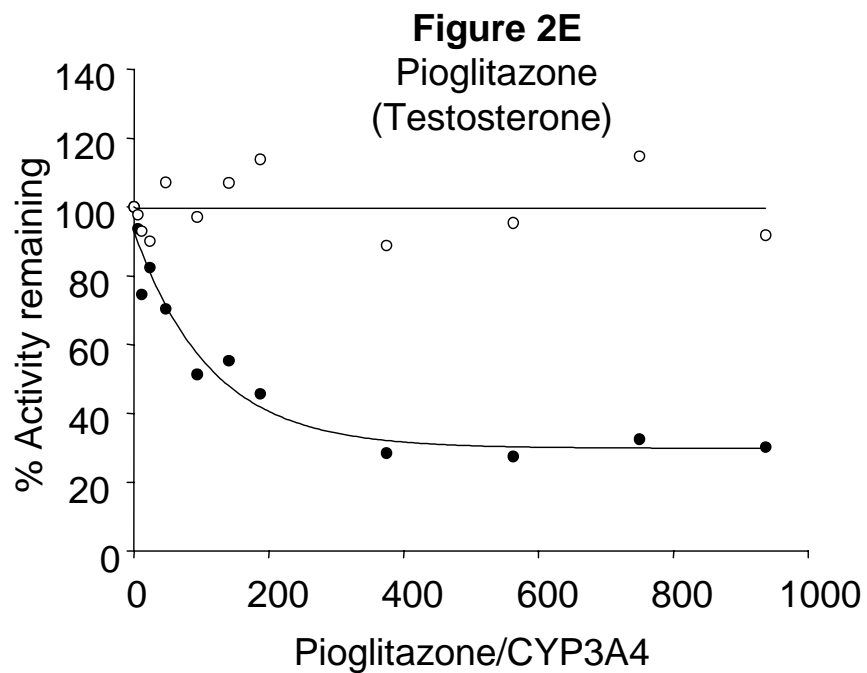


Figure 2G
Troglitazone

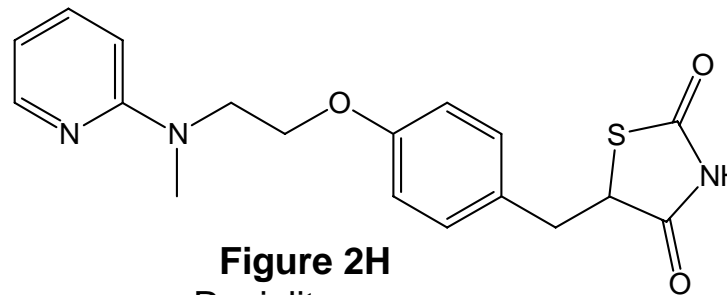


Figure 2H
Rosiglitazone

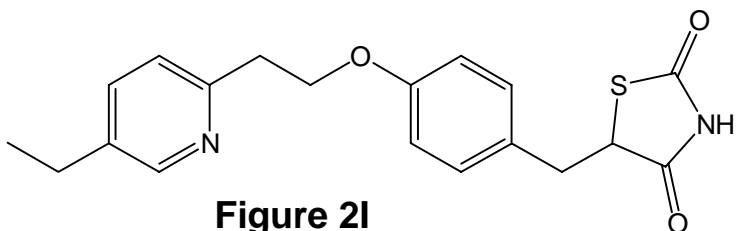


Figure 2I
Pioglitazone

Figure 3A

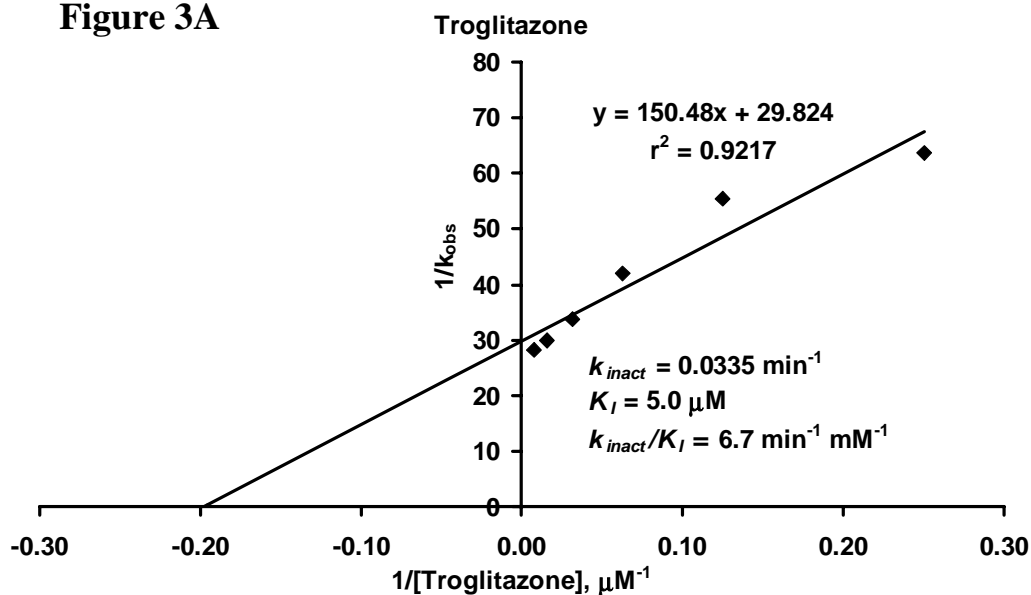


Figure 3B

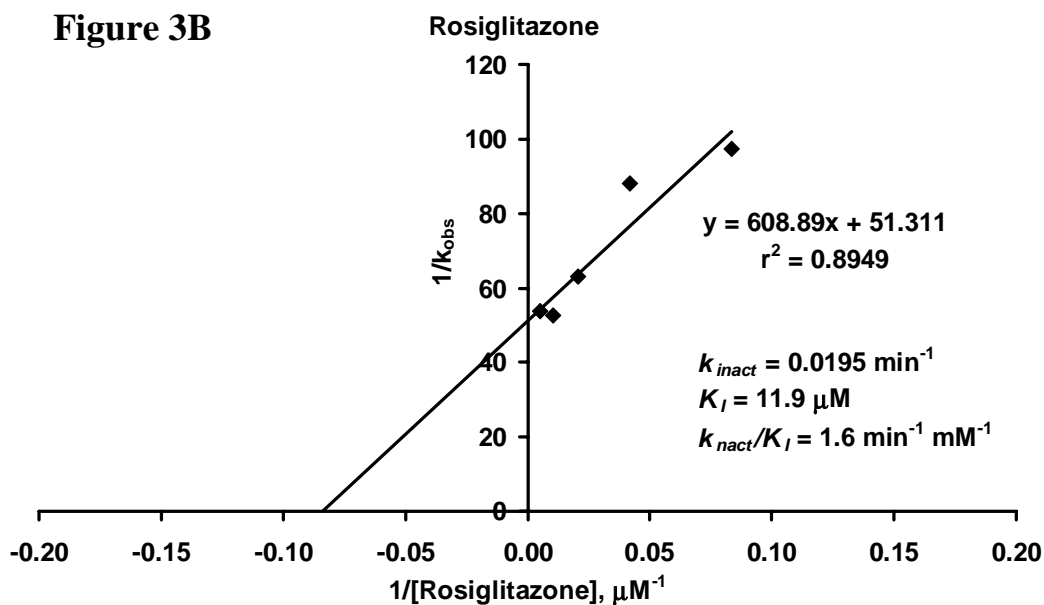


Figure 3C

