DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Selective time- and NADPH-dependent inhibition of human CYP2E1 by Clomethiazole

David M. Stresser, Elke S. Perloff, Andrew K. Mason, Andrew P. Blanchard, Shangara S. Dehal, Timothy P. Creegan, Ritu Singh, and Eric T. Gangl

Corning Life Sciences, Woburn, MA (DMS, TPC, RS), BD Biosciences, Woburn, MA (ESP, AKM, APB, SSD, ETG)

Running title: Selective inhibition of human CYP2E1 by Clomethiazole

Corresponding author:

David M. Stresser, Ph.D.

Corning Life Sciences

6 Henshaw Street

Woburn, MA 01801

Stresser5@gmail.com

T 847-935-4327

m 617-240-7737

Number of text pages: 11

Number of tables: 3

Number of figures: 6

Number of references: 38

Number of words in the Abstract: 226

Number of words the Introduction: 490

Number of words in the Discussion: 1497

List of nonstandard abbreviations:

DDI, drug-drug interaction(s)

CMZ, clomethiazole

HLM, human liver microsomes

IC_{50}, inhibitor concentration that produces a 50% reduction in product formation rate at a defined substrate-enzyme concentration

K_I, concentration of inhibitor giving half-maximal enzyme inactivation rate

K_i, dissociation constant for reversible inhibition

k_{inact}, the maximal inactivation rate constant

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

rCYP2E1, recombinant human CYP2E1 (CYP2E1 + OR + b5 Supersomes)

TDI, Time-dependent inhibition

$F_{m,CYP2E1}$, Fraction metabolized by CYP2E1

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The sedative clomethiazole (CMZ) has been used in Europe since the mid-1960s to treat insomnia and alcoholism. It has been previously demonstrated to reversibly inhibit human CYP2E1 *in vitro* and decrease CYP2E1-mediated elimination of chlorzoxazone in clinical studies. We have investigated the selectivity of CMZ inhibition of CYP2E1 in pooled human liver microsomes. In a reversible inhibition assay of the major drug-metabolizing cytochrome P450 isoforms, CYP2A6 and CYP2E1 exhibited IC_{50} values of 24 µM and 42 µM, respectively with all other isoforms exhibiting values > 300 µM. However, when CMZ was preincubated with NADPH and liver microsomal protein for 30 min prior to combining with probe substrates, more potent inhibition was observed for CYP2E1 and CYP2B6, but not CYP2A6 or other P450 isoforms. The substantial increase in potency of CYP2E1 inhibition upon preincubation enables the use of CMZ to investigate the role of human CYP2E1 in xenobiotic metabolism and provides advantages over other chemical inhibitors of CYP2E1. The K_I and k_{inact} values obtained with HLM-catalyzed 6-hydroxylation of chlorzoxazone were found to be 40 µM and 0.35 min^{-1}, respectively and similar to values obtained with recombinant CYP2E1 (41µM, 0.32 min^{-1}). The K_I and k_{inact} values along with other parameters were used in a mechanistic static model to explain published observations of profound inhibition of CYP2E1 *in vivo* in the absence of detectable CMZ in volunteers.

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Clomethiazole (CMZ, figure 1), is a sedative, hypnotic and anti-convulsant introduced into clinical practice in the 1960s and currently in use for management of restlessness, agitation, and insomnia in the elderly. It may also be used for treatment of acute alcohol withdrawal (Anonymous, 2016). In recent years, CMZ or its analogs have been investigated as a neuroprotectant in the treatment of ischemic stroke (Zingmark et al, 2003, Vandevrede et al, 2014). Although CMZ has been described as an inhibitor of human CYP2E1 both *in vitro* and *in vivo* (FDA, 2006; Gebhardt et al, 1997), the selectivity for inhibition of this enzyme has not been thoroughly investigated or reported.

CYP2E1 is an abundant hepatic P450 isoform known for its role in the metabolism of low molecular weight substrates including chemical carcinogens, organic solvents, and anesthetics (Gonzalez, 2007). Among non-anesthetic drugs, CYP2E1 participates in the metabolism of chlorzoxazone, acetaminophen, dapsone, eszopiclone, carbamazepine, felbamate, phenacetin and phenobarbital. Here we report that CMZ exhibits moderate reversible inhibition of CYP2A6 and CYP2E1, but is a selective and potent inhibitor of CYP2E1 upon preincubation of liver microsomes with NADPH. Therefore, we suggest that CMZ is an effective new tool to elucidate the role of CYP2E1 in reactions catalyzed by human liver microsomes and is advantageous over other less selective inhibitors, such as 4-methylpyrazole and diethyldithiocarbamate (Chang et al, 1994; Ono et al, 1996).

A profound decrease in CYP2E1 catalytic activity, as measured by chlorzoxazone clearance, has been observed after patients received single-dose CMZ (Gebhardt, 1997; Eap et al, 1998). Gebhardt et al (1997) proposed that the mechanism was in part due to reversible inhibition, but sustained inhibition was observed concurrent with undetectable levels of CMZ in blood. They speculated the latter could be accounted for by changes in gene transcription as had been proposed for similar observations in rat (Hu et al, 1994). In light of results reported here, we propose that time-dependent inactivation of CYP2E1 *in vivo* offers an alternative explanation for the findings in clinical studies, and are supported by results of a mechanistic static model predicting this outcome.

Potential mechanisms of CYP2E1 inactivation by CMZ include conversion to a reactive metabolite(s) that bind covalently to the heme or apoprotein or form a tight-binding inhibitory complex. Such mechanisms would be consistent with those proposed for other thiazole-containing compounds (Sevrioukova and Poulos, 2010; Obach et al,

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

2008; Rock et al, 2014; Subramanian et al, 2010). The chloro-ethyl side chain of CMZ may also be involved in the enzyme inactivation described here. For example, desaturation on the side chain could generate an electrophilic haloalkene species that may react with heme or apo-protein residue (Guengerich, 2001). The primary aims of the current work were to characterize the time- and NADPH inhibition of human CYP2E1 by CMZ, demonstrate that inhibition is highly selective for this enzyme, and illustrate using a mechanistic static model how these results could better explain past observations of CYP2E1 inhibition in humans.

Materials and Methods

Chemicals. Bupropion, S-mephenytoin, midazolam, 6-hydroxychlorzoxazone, 7-hydroxycoumarin-D5, acetamidophenol-[13C2 15N], hydroxybupropion, hydroxybupropion-[D6], desethylamodiaquine, desethylamodiaquine-[D3], 4´-hydroxydiclofenac, 4´-hydroxydiclofenac-[13C6], 4´-hydroxymephenytoin, 4´-hydroxymephenytoin-[D3], dextrorphan, dextrorphan-[D3], 1´-hydroxymidazolam , 1´-hydroxymidazolam-[13C3], 6ß-hydroxytestosterone, 6ß-hydroxytestosterone-[D7], NADPH regenerating system (concentrated solutions of NADP+, glucose-6-phoshate, glucose 6-phosphate dehydrogenase), CYP2E1+OR+b5 Supersomes (lot no. 4106001), insect cell control Supersomes and human liver microsomes (UltraPool™ HLM 150) pooled from 75 male and 75 female Caucasians donors were obtained from Corning Life Sciences (Tewksbury, MA). The 6-hydroxychlorzoxazone $D_2{}^{15}N$ was obtained from TLC Pharmachem (Aurora, ON). Phenacetin, amodiaquine, diclofenac, dextromethorphan, testosterone, acetamidophenol and 7-hydroxycoumarin and were purchased from Sigma Aldrich (St Louis, MO, USA).

Cytochrome P450 inhibition assays. All incubations were conducted in deep well polypropylene plates (Corning Life Sciences, Tewksbury, MA). For direct, reversible inhibition assays, several concentrations of clomethiazole (0.3, 1, 3, 10, 30, 100, 300, 600 µM) were incubated in 0.1 M potassium phosphate buffer, pH 7.4 containing 60 µM chlorzoxazone, 0.05-0.1 mg/mL HLM and an NADPH regenerating system (1.3 mM NADP+, 3.3 mM glucose6-phosphate, 0.4 U ml^{-1} glucose 6-phosphate dehydrogenase, 3.3 mM magnesium chloride) in a 400 µL volume. After a 10 min warm up period, reactions were initiated with addition of HLM. After the 5 min incubation, reactions were terminated by the addition of 100 µL of 200 nM 6-hydroxychlorzoxazone $D_2{}^{15}N$ in acetonitrile containing 0.1%

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

formic acid. For K_i experiments, conditions were similar except chlorzoxazone substrate concentrations were 15, 30, 60, 120, 240 µM and CMZ inhibitor concentrations were 0 (water vehicle), 12.5, 25, 50, 100 and 200 µM.

IC_{50} shift assays. To screen for TDI, incubations were conducted with and without a 30 minute preincubation step. With CYP2E1, an additional 10 min preincubation step was incorporated as a preliminary evaluation of the rate of inactivation (Perloff et al, 2009). To confirm NADPH dependence of inhibition of CYP2E1, additional preincubation experiments were conducted for 30 min with and without an NADPH regenerating system. In a final volume of 400 µL, clomethiazole at varying concentrations [e.g. 0 (water vehicle), 0.1, 0.3, 1.0, 3.0, 10, 30, 100, 300 µM] was preincubated with 10X HLM (or 5X for CYP2C19 enzyme activity) at 37ºC with an NADPH regenerating system in 0.1 M potassium phosphate buffer, pH 7.4. At the end of the preincubation period, 40 µL of the reaction mix was diluted into a secondary incubation containing 360 µL of 0.1 M potassium phosphate buffer, pH 7.4 containing an NADPH regenerating system and a CYP-selective probe substrate at a concentration at or near the K_m. For target P450s, concentrations of substrates and HLM protein were as follows: CYP1A2, phenacetin, 40 µM, 0.2 mg/mL; CYP2A6, coumarin, 1.5 µM, 0.05 mg/mL; CYP2B6, bupropion, 80 µM, 0.1 mg/mL; CYP2C8, amodiaquine, 1.5 µM, 0.02 mg/mL; CYP2C9, diclofenac, 5 µM, 0.05 mg/mL; CYP2C19, (S)-mephenytoin, 40 µM, 0.3 mg/mL; CYP2D6, dextromethorphan, 5 µM, 0.1 mg/mL; CYP3A4, midazolam, 3 µM, 0.02 mg/mL; CYP3A4, testosterone, 50 µM, 0.05 mg/mL. Incubations were carried out for either 5 or 10 min, stopped by addition of 100 µL 0.1% formic acid in acetonitrile containing an internal standard, centrifuged prior to analysis of metabolites by LC/MS/MS (Perloff et al, 2009). For CYP2A6, incubations were carried out for 5 min and were stopped by the addition of 100 µL of 7-hydroxycoumarin-D_5 in acetonitrile with 0.1% formic acid. For CYP2E1, the concentration of HLM protein was 1.0 mg/mL in the preincubation (0.1 mg/mL in secondary incubation) and the probe substrate chlorzoxazone was 60 µM. After 5 min of incubation the reaction was stopped by the addition of 100 µL of 6-hydroxychlorzoxazone $D_2{}^{15}N$ in acetonitrile with 0.1% formic acid.

K_I and k_{inact} assays. To determine K_I and k_{inact} values for CMZ, 0, 3, 10, 30, 100 and 300 µM CMZ was preincubated in 0.1 M potassium phosphate buffer, pH 7.4 with HLM (1.0 mg/mL) or CYP2E1 Supersomes (57 pmol/mL, normalized to 1.0 mg/mL with insect cell control protein) and a NADPH-regenerating system for 0, 2, 4, 6, 8, and 10 min followed by a 10-fold dilution into a secondary incubation containing 0.1 M potassium phosphate buffer, NADPH-regenerating system and chlorzoxazone (300 µM) for 5 min. Incubations were terminated and processed as described earlier for CYP2E1.

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Data analysis. IC_{50} values were determined by nonlinear regression using a four parameter logistic fit model (XLfit, model 205), with max and minimum values locked at 100 and 0, respectively. The selection of 0 as minimum value enabled a more robust comparison of inhibition potency. K_i values were determined using Sigma Plot, v8.0 equipped with Enzyme Kinetics Module v. 1.1. Four models were compared (pure competitive, mixed, pure noncompetitive, uncompetitive), and the choice of best fit was determined by the Akaike Information Criterion (AIC), visual inspection of the Michaelis-Menten and Eadie-Hofstee plots and also R^2 values. The k_{obs} values were determined for each concentration of CMZ to enable determination of k_{inact} and K_I using equation 1.

$$k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_I + [I]} \qquad \text{(eq 1.)}$$

Where:

k_{obs} is the observed rate constant for inactivation

k_{inact} is the maximal inactivation rate constant

[*I*] is the concentration of inactivator in the primary incubation

K_I is the concentration of inactivator at which the rate of inactivation is half maximal

The raw data at each time point was normalized to the corresponding 0 µM control and the normalized data were then transformed to the natural log of the percent chlorzoxazone 6-hydroxylase activity remaining and plotted versus the preincubation time. The slope of each line was determined by linear regression and represents k_{obs}, the observed rate constant for inactivation at a specified concentration of CMZ. Only pre-incubation time points that defined a straight line were used to determine slope; at the higher concentrations and longer preincubation time points, the rate of inactivation slowed and no longer defined initial inactivation rates. The values of k_{obs} were then plotted versus CMZ concentration. The parameters K_I and k_{inact} were determined by non-linear regression using XL-fit (v. 5.3, IDBS, Guildford, UK) software (model 250, with the value of n constrained to 1).

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Prediction of drug-drug interaction. The magnitude of a potential drug-drug interaction for CMZ and chlorzoxazone was evaluated using a static model incorporating both reversible inhibition and time-dependent inactivation (Fahmi et al, 2008) as modified in the FDA guidance pertaining to drug-drug interactions (FDA, 2012). As CMZ is not known to be an inducer, we have omitted the variable representing induction. In addition, no variable to model intestinal inhibition is included, as CYP2E1 and CYP2A6 are generally considered to be undetectable in intestine. In this model, the DDI is expressed as the ratio of area under the exposure time curve in the presence ($AUC'_{p.o.}$) and absence ($AUC_{p.o.}$) of a pharmacokinetic drug-drug interaction:

$$\frac{AUC'_{p.o.}}{AUC_{p.o.}} = \left(\frac{1}{[A \times B] \times f_m + (1 - f_m)}\right) \quad \text{(eq 2.)}$$

Where *A* is the term for enzyme inactivation in the liver:

$$A = \frac{k_{deg}}{k_{deg} + \frac{[I] \times k_{inact}}{[I] + K_I}} \quad \text{(eq 3.)}$$

The term k_{deg} is the degradation rate constant for CYP2E1 in the liver. In eq. 2, *B* is the term for reversible inhibition in the liver, defined as:

$$B = \frac{1}{1 + \frac{[I]}{K_i}} \quad \text{(eq 4.)}$$

Where $[I] = f_{u,b} \times ([I]_{max,b} + F_a \times K_a \times Dose/Q_h)$; $f_{u,b}$ is the unbound fraction in blood; $[I]_{max,b}$ is the maximal total (free and bound) perpetrator concentration in the blood at steady state; F_a is the fraction absorbed after oral administration; K_a, is the first order absorption rate constant in vivo; and Q_h is hepatic blood flow (97 L/hr/70 kg); The term f_m in eq 2 refers to the fraction metabolized by CYP2E1.

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Results

When CMZ was co-incubated with HLM and the probe substrate chlorzoxazone at a concentration of approximately K_m, IC_{50} value for CYP2E1 was found to be 42 ± 8.9 µM (Table 1, Figure 2). Using multiple chlorzoxazone concentrations bracketing the K_m, and CMZ concentrations of 12.5, 25, 50, 100 and 200, a K_i value of 21 ± 1.7 µM (Table 1) was obtained. When CMZ was incubated with HLM in the presence of NADPH for 10 or 30 min, prior to a 10-fold dilution into a secondary incubation containing chlorzoxazone, IC_{50} values shifted lower to 0.96 to 1.03 µM, respectively. To investigate the time-dependent inhibition properties of CMZ further, K_I and k_{inact} values were determined using HLM and found to be 40 µM and 0.35 min^{-1} , respectively (Table 2, Figure 3). These values were derived from k_{obs} values determined from the linear portion of the natural log-transformed 6-hydroxychlorzoxazone activity loss observed in the preincubation phase (shown in Figure 4 shown without log-transformation of activity to better illustrate the time and concentration dependence of inhibition response in a potential phenotyping experiment). Additional K_I and k_{inact} experiments were carried out with rCYP2E1 and values were determined to be 41 µM and 0.32 min^{-1}, respectively (Table 2). In addition, time-dependent inhibition of rCYP2E1 was found to be dependent on preincubation with NADPH (data not shown). The selectivity of CMZ inhibition of CYP2E1 was evaluated by comparing inhibition response with eight other P450 enzymes (Figure 5). Only CYP2A6 and CYP2E1 exhibited inhibition > 50% at concentrations up to 300 µM. The IC_{50} value for CYP2A6 was determined to be 24 µM (Table 1, Figure 2, panel B, blue), slightly lower than values for CYP2E1. Less potent inhibition was found with other enzymes (Figure 5) including CYP2B6, CYP2C8, CYP2C19 and CYP3A4 (with testosterone as a substrate) whereas other enzymes (CYP1A2, CYP2C9 and CYP2D6) were marginally inhibited or weakly activated (CYP3A4 with midazolam as substrate). However, when CMZ was preincubated with NADPH and microsomal protein for 30 min prior to combining with probe substrates, much more potent inhibition was observed for CYP2E1 (> 40-fold shift in IC_{50}, Table 1). Therefore, CMZ inactivated CYP2E1 (Table 1, Table 2, Figure 5) in a time- and NADPH-dependent manner. We also observed that CYP2B6 exhibited TDI, as the percent inhibition response increased by approximately 10-fold (Table 1) after preincubation with NADPH for 30 min. While the absence of time-dependent inhibition of CYP2A6 could not be ruled out from these data, it is clear that any effect is weak (1.3-fold shift in IC_{50}, Table 1) and does not meet the criteria of a 1.5-fold shift to indicate TDI as suggested by Grimm et al (2009).

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Prediction of CMZ interaction with CYP2E1 substrates. Model-based predictions of drug-drug interactions are now recommended by regulatory agencies. As a follow up analysis to the "basic" model for an interacting drug described in regulatory guidance (FDA, 2012), a mechanistic static pharmacokinetic model is suggested as a precursor to determine whether a clinical drug-drug interaction study is required. In this model, the net effect of enzyme induction, reversible inhibition and time-dependent inhibition are incorporated, both in liver and gut. We invoked the mechanistic static model (line 676 in the 2012 FDA guidance) to predict a drug interaction resulting from oral administration of CMZ and an object drug metabolized by CYP2E1. As CYP2E1 is generally considered to be absent in gut, and CMZ is not known to be an inducer, we excluded these terms in our modified version of the static model (equation 2), incorporating hepatic reversible inhibition and time-dependent inhibition only. Other parameter inputs were obtained from literature values (Supplemental Table 1). Figure 6 provides the results of this analysis. The sensitivity of the fold-increase in AUC ratio, as the inputs of dose and $F_{m,CYP2E1}$ are altered, is shown. For this analysis, we assumed [I] increased linearly with dose, up to 384 mg CMZ, equivalent to two 300 mg capsules of clomethiazole edisylate. Although oral doses exceeding 2 g/day are used in alcohol cessation treatment in a hospital setting, it was apparent that modeling higher doses had negligible impact on the DDI prediction. The predicted AUC change incorporating reversible inhibition only (Figure 6, panel A) or both reversible and TDI was examined (Figure 6, panel B). From this analysis, it is expected that enzyme inactivation by CMZ would have a much greater impact on AUC change than pure reversible inhibition.

Discussion

Our results confirm previous observations that CMZ is a direct inhibitor of human CYP2E1 when co-incubated with the probe substrate chlorzoxazone. Gebhardt et al (1997) showed a K_i value of 12 μM which is similar to the mean K_i value of 21 μM reported here. Previously unreported is the finding that CMZ inactivates CYP2E1 in a time- and NADPH-dependent manner. Inactivation in HLM by CMZ was relatively efficient as determined by k_{inact}/K_I ratio of 8.8 μL min^{-1} $nmol^{-1}$ (Table 2) and this was primarily a function of its relatively high k_{inact} value [similar to values reported for tienilic acid/CYP2C9; ticlopidine/CYP2B6 and ritonavir/CYP3A (Obach et al, 2007)]. Results using rCYP2E1 gave essentially identical values as those found with HLM (Table 2) demonstrating CYP2E1 inactivation occurs without requirement of metabolism by other CYPs known to metabolize CMZ (Centerholt et al, 2003).

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

The selectivity of CMZ inhibition of CYP2E1 has been partially investigated *in vitro* and *in vivo*. Gebhardt et al (1997) reported that CYP1A2 and CYP3A were not inhibited *in vitro* and Mönig et al (1993) observed tolbutamide elimination was unaffected in volunteers, indicating no inhibition of CYP2C9. Our results confirm and extend these findings. In addition to the aforementioned enzymes, other enzymes that were refractory to inhibition (IC_{50} values > 300 μM) were CYP2B6, CYP2C8, CYP2C19 and CYP2D6. We found that CYP2A6 was inhibited to a similar extent as CYP2E1 and therefore, under conditions of reversible inhibition, CMZ is not ideal as an inhibitor probe of CYP2E1. However, when CMZ was evaluated as a time-dependent inhibitor in our panel, the inhibition of CYP2E1 was greatly enhanced, whereas inhibition of CYP2A6 essentially did not increase. Therefore, CMZ can be used a mono-selective, chemical inhibitor to probe the role of CYP2E1 metabolism *in vitro* using the appropriate experimental conditions. To obtain selectivity, we recommend preincubating 1 mg/mL HLM with NADPH for 10 min at a nominal concentration of 30 μM, followed by a 10-fold dilution into an incubation containing the substrate under investigation. A non-dilution preincubation method is not recommended, as a lower concentration of CMZ (e.g. 3 μM) would be needed to dissociate the reversible CYP2A6 inhibition component and a much longer preincubation period (e.g. 60 min) would probably be needed to suitably inactivate CYP2E1. With a longer preincubation period, non-specific and CMZ-independent activity loss of other CYPs may occur.

The fraction of CMZ unbound to microsomes was not determined in the current study, but this parameter may be estimated based on lipophilicity. Using a calculated log D value of 2.12 for CMZ (Yoshida and Topliss, 2000), and the algorithm described by Austin et al (2002), the fu_{mic} at 4, 2 and 1 mg/mL is 0.29, 0.46 and 0.63. Accordingly, if HLM concentrations greater than 1 mg/mL are used, higher concentrations of CMZ may be needed to achieve a suitable unbound concentration that retains adequate CYP2E1 inhibition. Clomethiazole is freely water soluble, therefore stock solutions may be prepared without using organic solvents, known to be particularly inhibitory to CYP2E1 (Chauret et al, 1998). In our laboratory, we prepare 20 mM stock solutions in water. Some mild heat and sonication may be required to obtain full dissolution.

From Figure 2, it is evident that CMZ did not completely inactivate chlorzoxazone 6-hydroxylase activity, even after a 30 min preincubation with 600 μM CMZ. One explanation for this is that CMZ fails to significantly inhibit the

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

high affinity, CYP1A2 contribution to chlorzoxazone 6-hydroxylase activity (Yamamura et al, 2015). If this is the case, the extent of inhibition observed suggests essentially a complete inactivation of the predominant CYP2E1 component of the reaction.

Relative to reversible inhibition, the inactivation of CYP enzymes is a mechanism more prone to elicit drug-drug interactions. This is because restoration of catalytic activity requires elimination of the perpetrating drug as well as *de novo* synthesis of the affected enzyme, a process that may take days. Recent guidance documents from regulatory agencies have incorporated mechanistic static models (developed over several years by multiple researchers) designed to predict the extent of changes in AUC caused by mechanism-based inhibitors. Using the model in equation 2 along with parameter values estimated or obtained experimentally (Supplemental Table 1), CMZ appears to be capable of causing significant increases in the AUC of CYP2E1 substrates, even at very low, subtherapeutic doses ($\leq$ 10 mg/day) of CMZ. As depicted (Figure 6), the extent of the interaction is highly dependent on the $F_{m,CYP2E1}$. By contrast, much less dependency was found when values of k_{deg} for CYP2E1 representing the range observed in 10 patients [0.000145 to 0.000477 min^{-1}, (Emery et al, 1999)] was input to the model (data not shown). Our model included both TDI (eq. 3) and reversible inhibition (eq. 4). However, as can be seen in panel B of Figure 6, the predicted AUC changes were highly dependent on the inactivation term (eq. 3). These simulations support the inactivation component as the primary contributor to the changes in CYP2E1 activity observed in the clinic.

The pharmacokinetics of CMZ is well documented by multiple clinical studies (Rätz et al, 1999; Centerholt et al, 2003; Wilby and Hutchinson, 2004 and references therein). With oral dosing, peak plasma concentrations occur approximately 1 h after administration with an elimination half-life of about 4 h (Rätz et al, 1999). Clearance is hepatic blood-flow limited and occurs primarily through metabolism by CYP2A6, CYP3A4/5 and CYP2B6 (Centerholt et al, 2003) and CYP2E1 (data not shown). Compared to drug-free volunteers, the AUC of chlorzoxazone, given in a 250 mg dose, increased 3.4-fold in alcoholic patients receiving daily 1.2 to 2.4 g CMZ at least 1 to 3 days prior to chlorzoxazone (Eap et al, 1998). The AUC change coincided with an approximate 81% decrease in 6-hydroxychlorzoxazone/chlorzoxazone ratio, a measure of CYP2E1 activity. Gebhardt et al (1997) found that the 6-hydroxychlorzoxazone/chlorzoxazone ratio was reduced 85-94% in alcoholic patients who received

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

therapeutic doses of CMZ (0.6 to 2.3 g/d) 0.5-12 h prior to chlorzoxazone. In another arm of that study, healthy controls received either 192 mg or 384 mg capsules, followed by administration of 500 mg of chlorzoxazone 12, 36, 60, and 84 h later. In these patients, there was a profound (83%) decrease in the 6-hydroxychlorzoxazone/chlorzoxazone ratio. Moreover, 6-hydroxychlorzoxazone/chlorzoxazone ratios did not return to initial levels until 36 h for the 192 mg dosed patients and until 60 h for the 384 mg dosed patients. Additionally, clomethiazole was not detected in blood at 12 h post dosing in either patient group. The observation of undetectable CMZ concurrent with highly depressed CYP2E1 activity is consistent with inactivation of this enzyme *in vivo*, and agrees well with our findings of efficient *in vitro* inactivation of CYP2E1. Concordant with our modeling exercise, the extent of CYP2E1 activity loss seemed to be dose-independent at 192 mg/day or more. In other words, the loss of CYP2E1 activity observed in patients taking 192 mg/day was similar to patients taking 2.4 g/day.

The mechanism of CYP2E1 inactivation was not thoroughly investigated in the current study. Inactivation was found to be metabolism-dependent, required NADPH and did not require metabolism by P450 isoforms other than CYP2E1. A possible mechanism of inactivation includes conversion of CMZ to reactive metabolites that bind covalently to heme or apoprotein. A non-covalent, metabolic intermediate complex could also form after NADPH-dependent conversion to a tight-binding metabolite(s). It is interesting to note that CMZ contains a thiazole functional group in common with potent CYP3A4 inhibitors cobicistat and ritonavir and compounds known to undergo metabolic activation and covalent binding to microsomal protein, including ritonavir, sudoxicam, meloxicam and a series of AKT inhibitors (Obach et al, 2008; Rock et al, 2014; Subramanian et al, 2010). It has been postulated that initial events leading to metabolic activation of the thiazole, and subsequent covalent binding are epoxidation of the C4-C5 bond (Subramanian et al, 2010) or sulfoxidation (Rock et al, 2014). Although ritonavir exhibits mechanism-based inhibition (Rock et al, 2014), metabolism-independent tight-binding has been suggested as a predominant means of CYP3A4 inactivation, whereby the thiazole nitrogen coordinates to both ferric and ferrous forms of the enzyme, decreases heme redox potential and hinders electron transfer from cytochrome P450 reductase (Sevrioukova and Poulos, 2010). The analogous mechanism for CMZ and CYP2E1 would be less operative here because the reversible inhibition constant was 21 μM (Table 1) and the strict requirement for NADPH to elicit potent inhibition. Accordingly, metabolites of CMZ (Centerholt et al, 2003) might be more apt to fit this model. The chloro-ethyl side chain of CMZ may also be involved in inactivation in a mechanism similar to

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

that observed with alkanes (Guengerich, 2001). For example, desaturation on the chloroethyl side chain could yield an electrophilic haloalkene species that could react with heme or apo-protein residue. This mechanism might be more likely than epoxidation of C4-C5 bond discussed above which is sterically hindered by the chloro-ethyl side chain. Together, multiple mechanisms of metabolism-dependent inactivation of CYP2E1 (and CYP2C19) could be occurring. Future investigations into the mechanism should be facilitated by the relative simplicity of the CMZ structure.

Conclusions

Data presented here demonstrate that under incubation conditions described herein, CMZ is an efficient and selective time- and NADPH-dependent inhibitor of CYP2E1 *in vitro*. The selectivity was demonstrated by measuring changes in P450 isoform selective activity assays in reversible and time-dependent inhibition assays. We suggest that CMZ represents an effective and advantageous tool to phenotype CYP2E1 catalytic activity *in vitro*. In addition, our findings help explain previous clinical observations of profound changes in chlorzoxazone metabolism, and also suggest that CMZ may find use as a probe for inhibition of CYP2E1 *in vivo*, similar to disulfiram (Kharasch et al, 1993). Finally, our data suggest the need for heightened awareness for potential dose adjustments and/or patient monitoring when CMZ is given prior to drugs that are CYP2E1 substrates.

Acknowledgements

The authors thank Adrian Fretland at AstraZeneca and Amit Kalgutkar at Pfizer for their critical review and commentary during the preparation of the manuscript.

Authorship Contribution

Participated in research design: Stresser, Perloff, Gangl, Singh

Conducted experiments: Mason, Blanchard, Dehal, Gangl, Creegan

Performed data analysis: Stresser, Perloff, Singh, Mason, Gangl, Dehal

Wrote or contributed to the writing of the manuscript: Stresser, Perloff

References

Anonymous. Electronic Medicines Compendium (2016) http://www.medicines.org.uk/emc/medicine/25188. accessed January 4, 2016.

Austin RP, Barton P, Cockroft SL, Wenlock MC, and Riley RJ (2002) The influence of nonspecific microsomal binding on apparent intrinsic clearance, and its prediction from physicochemical properties. Drug Metab Dispos 30: 1497-1503

Centerholt C, Ekblom M, Odergren T, Borgå O, Popescu G, Molz, K-H, Couturier A, Weil A (2003) Pharmacokinetics and sedative effects in healthy subjects and subjects with impaired liver function after continuous infusion of clomethiazole. Eur J Clin Pharmacol. 59:117–122.

Chang TKH, Gonzalez FJ and Waxman DJ (1994) Evaluation of triacetyloleandomycin, α-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. Arch. Biochem. Biophys. 311:437-442.

Chauret N, Gauthier A, Nicoll-Griffith DA (1998) Effect of common solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes. Drug Metab Dispos 26:1–4

Eap CB, Schnyder C, Besson J, Savary L, Buclin T (1998) Inhibition of CYP2E1 by chlormethiazole as measured by chlorzoxazone pharmacokinetics in patients with alcoholism and in healthy volunteers. Clin Pharmacol Ther 64:52–57

Emery MG, Jubert C, Thummel KE, Kharasch ED (1999) Duration of cytochrome P-450 2E1 (CYP2E1) inhibition and estimation of functional CYP2E1 enzyme half-life after single-dose disulfiram administration in humans. J Pharmacol Exp Ther 291:213–219.

Fahmi OA, Maurer TS, Kish M, Cardenas E, Boldt S, and Nettleton D (2008) A combined model for predicting CYP3A4 clinical net drug-drug interaction based on CYP3A4 inhibition, inactivation, and induction determined in vitro. Drug Metab Dispos 36:1698–1708.

FDA (2006) Draft Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling Recommendations. FDA, Silver Spring, MD

FDA (2012) Draft Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling Recommendations. FDA, Silver Spring, MD

Gebhardt AC, Lucas D, Menez J-F and Seitz HK (1997) Chlormethiazole inhibition of cytochrome P450 2E1 as assessed by chlorzoxazone hydroxylation in humans. Hepatology 26:957–961

Gonzalez FJ (2007) The 2006 Bernard B. Brodie Award Lecture: CYP2E1. Drug Metab Dispos. 1:1-8

Gorski JC, Jones DR, Wrighton SA, Hall SD (1997) Contribution of human CYP3A subfamily members to the 6-hydroxylation of chlorzoxazone. Xenobiotica 27:243–256.

Green AR (1998) Clomethiazole (Zendra) in acute ischemic stroke: basic pharmacology and biochemistry and clinical efficacy. Pharmacol Ther 80:123–147

Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KH, Lu C, Nomeir AA, Seibert E, Skordos KW, et al (2009) The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the pharmaceutical research and manufacturers of America. Drug Metab Dispos 37:1355–1370

Guengerich FP (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol 14:611–650

Hu Y, Mishin V, Johansson I, von Bahr C, Cross A, Ronis MJ, Badger TM, Ingelman-Sundberg (1994) Chlormethiazole as an efficient inhibitor of cytochrome P450 2E1 expression in rat liver. J Pharmacol Exp Ther. 269:1286-91

Jostell K-G, Agurell S, Allgén L-G, Kuylenstierna B, Lindgren JE, Åberg G, Österlöf G (1978) Pharmacokinetics of clomethiazole in healthy adults. Acta Pharmacol Toxicol 43:180–189

Kharasch ED, Thummel KE, Mhyre J, Lillibridge JH (1993) Single-dose disulfiram inhibition of chlorzoxazone metabolism: a clinical probe for P450 2El. Clin Pharmaco1 Ther. 53:643-650.

Liao M, Kang P, Murray BP and Correia MA (2010) Cytochrome P450 degradation and its clinical relevance. In "Enzyme inhibition in drug discovery and development: The good and the bad (LuC and Li AP, eds). John Wiley & Sons, Hoboken, NJ.

Mather LE, Runciman WB, Ilsley AH, Thomson KR and Goldin AR (1981) Direct measurement of chlormethiazole extraction by liver, lung and kidney in man. Br. J. Clin. Pharmacol 12:319-325

Monig H, Back DJ, Heidermann HT, Ohnhaus EE, Brockmann B and Schulte HM (1993) Effect of chlormethiazole on hepatic mono-oxygenases activity in vivo. Eur. J. Clin. Pharmacol.44: 203–204.

Newton DJ, Wang RW and Lu AYH (1995) Evaluation of specificities in the in vitro metabolism of therapeutic agents by human liver microsomes. Drug Metab Dispos. 23:154-158.

Obach RS, Kalgutkar AS, Ryder TF and Walker GS (2008) In vitro metabolism and covalent binding of enol-carboxamide derivatives and anti-inflammatory agents sudoxicam and meloxicam: insights into the hepatotoxicity of sudoxicam. Chem. Res. Toxicol. 21:1890-1899

Obach RS, Walsky RL, Venkatakrishnan K (2007) Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug–drug interactions. Drug Metab Dispos 35, 246–255.

Ono S, Hatanaka T, Hotta H, Satoh T, Gonzalez FJ and Tsutsui M (1996) Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of in vitro metabolism using cDNA-expressed human P450s and human liver microsomes. Xenobiotica. 26:681-693

Perloff ES, Mason AK, Dehal SS, Blanchard AP, Morgan L, Ho T, Dandeneau A, Crocker RM, Chandler CM, Boily N, Crespi CL, and Stresser DM (2009) Validation of a Cytochrome P450 Time Dependent Inhibition Assay: A Two Time Point IC50 Shift Approach Facilitates kinact Assay Design. Xenobiotica. 39:99-112

Rätz AE, Schlienger RG, Linder L, Langewitz W, Haefeli WE (1999). Pharmacokinetics and pharmacodynamics of clomethiazole after oral and rectal administration in healthy subjects. Clin Ther. 21:829–840

Rock BM, Hengel SM, Rock DA, Wienkers LC, Kunze KL (2014) Characterization of Ritonavir-Mediated Inactivation of Cytochrome P450 3A4. Mol. Pharmacol. 86: 665– 674

Sevrioukova IF, Poulos TL (2010) Structure and mechanism of the complex between cytochrome P4503A4 and ritonavir. Proc Natl Acad Sci USA 107: 18422-18427

Subramanian R, Lee MR, Allen JG, Bourbeau MP, Fotsch C, Hong FT, Tadesse S, Yao G, Yuan CC, and Surapaneni S et al. (2010) Cytochrome P450-mediated epoxidation of 2-aminothiazole-based AKT inhibitors: identification of novel GSH adducts and reduction of metabolic activation through structural changes guided by in silico and in vitro screening. Chem Res Toxicol 23:653–663

Vandevrede L, Tavassoli E, Luo J, Qin Z, Yue L, Pepperberg DR, Thatcher GR (2014) Novel analogues of chlormethiazole are neuroprotective in four cellular models of neurodegeneration by a mechanism with variable dependence on GABAA receptor potentiation. Br J Pharmacol. 171:389–402

Wilby MJ, Hutchinson PJ (2004) The pharmacology of chlormethiazole: A potential neuroprotective agent? CNS Drug Rev. 10:281-294.

Yamamura Y, Koyama N, Umehara K (2015) Comprehensive kinetic analysis and influence of reaction components for chlorzoxazone 6-hydroxylation in human liver microsomes with CYP antibodies. Xenobiotica, 45:353-360.

Yoshida F, Topliss JG (2000) QSAR model for drug human oral bioavailability. J Med Chem. 43: 2575-2585

Zingmark PH, Ekblom M, Odergren T, Ashwood T, Lyden P, Karlsson MO, Jonsonn EN (2003). Population pharmacokinetics of clomethiazole and its effect on the natural course of sedation in acute stroke patients. Br J Clin Pharmacol. 56:173–83.

Unnumbered footnotes:

Portions of this work were previously presented at the 2010 International Society of Study of Xenobiotics (Istanbul, Turkey)

Clomethiazole is also known as [5-(2-chloroethyl) 4-methylthiazole], chlormethiazole, Distraneurin®, Hemineurin®, Heminevrin®)

ESP, AKM, APB, SSD, ETG were employed by Discovery Labware, Inc, a business unit of BD Biosciences, Woburn, MA at the time of their experimental contributions to the work. Discovery Labware, Inc was subsequently acquired by Corning Life Sciences.

Address reprint requests to David Stresser, Corning Life Sciences, 6 Henshaw st, Woburn, MA 01801; Stresser5@gmail.com

Numbered footnotes:

[1] – David M. Stresser, Current affiliation: AbbVie Inc., 1 N. Waukegan Rd, North Chicago, IL

[2] – Elke S. Perloff, Current address: Somerville, MA

[3] – Andrew K. Mason, Current affiliation: Dept Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY

[4] – Andrew P. Blanchard, Current affiliation: Mylan Institutional, Sugar Land, TX

[5] – Shangara S. Dehal, Current affiliation: Cardinal Health, Woburn, MA

[6] – Eric T. Gangl, Current affiliation: AstraZeneca, Waltham, MA

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Structure of clomethiazole

Figure 2. Effect of preincubation of clomethiazole with HLM and NADPH on CYP2E1-catalyzed chlorzoxazone 6-hydroxylase activity (A) and CYP2A6-catalyzed coumarin 7-hydroxylase activity (B). Data from four (CYP2E1) or two (CYP2A6) independent experiments were combined to construct the global curve fit for each condition. For each panel, data represent results obtained with (green triangles) and without (blue circles) preincubation, respectively.

Figure 3. Determination of K_I and k_{inact} parameters for the inactivation of chlorzoxazone 6-hydroxylation by clomethiazole in pooled HLM. Each data point represents the mean of duplicates and data from two independent experiments are shown. The K_I and k_{inact} values obtained with HLM and rCYP2E1 are shown in Table 1.

Figure 4. Loss of chlorzoxazone 6-hydroxylation activity observed after preincubation of CMZ at the concentrations indicated with 1 mg/mL HLM in the presence of an NADPH regenerating system. The 6-hydroxychlorzoxazone formation was determined after a 10-fold dilution into a secondary incubation containing 300 μM chlorzoxazone. Data points are the means of two experiments conducted on independent days.

Figure 5. Effect of CMZ on P450 isoform activity in pooled HLM. Enzyme activities were conducted as described in the methods and in Perloff et al (2009). Panel A, direct, reversible inhibition assay; Panel B, Time-dependent inhibition assay, where CMZ was incubated with HLM and NADPH for 30 min prior to 10-fold dilution (except for CYP2C19, which was 5-fold) into a secondary incubation containing probe substrate. The concentrations evaluated are shown in the figure legend and are shown in units of μM. For the TDI assay, concentrations spaced by the arrow represent those in the preincubation and secondary incubation, respectively. Values represent means of duplicate incubations and the global mean range among duplicate values was 0.047.

Figure 6. Simulated effect of CMZ on AUC ratio of plasma concentrations of a CYP2E1 substrate with different levels of $F_{m,CYP2E1}$ deploying a mechanistic static model (eq. 2) incorporating both TDI and reversible inhibition (Panel A) or reversible inhibition only (Panel B); in the latter simulation, the *A* term in eq.2 is fixed at a value of 1. The dose range modeled is 0.1 mg to 384 mg with the upper end of the range based on the prescribing information used to treat restlessness and agitation.

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Summary of CMZ inhibition parameters for CYP2E1, CYP2A6 and CYP2B6 [a]

Enzyme	IC_{50} value (μM)	K_i value (μM)	IC_{50} value, 10 min preincubation[b] (μM)	IC_{50} value, 30 min preincubation (μM) [b]
CYP2E1	42 ± 8.9	21 ± 1.7	0.96 ± 0.27	1.03 ± 0.22
CYP2A6	24 ± 4.8	ND[c]	ND	19 ± 5
CYP2B6	> 300 [d]	ND	ND	> 30 [e]

[a] - Each value represents the mean ± standard deviation of 4 (CYP2E1, IC_{50} value with and without 30 min preincubation) or range of 2 (all other conditions) independent experiments

[b]- Preincubation of CMZ with HLM and NADPH. Preincubation of CMZ at concentrations up to 100 μM with HLM in the absence of NADPH for 10 or 30 min prior to addition of 60 μM chlorzoxazone substrate yielded IC_{50} values > 10 μM (the concentration of CMZ after 10-fold dilution), with maximum inhibition of 25%.

[c]- ND, Not determined

[d]- 45 ± 0.8 (n = 2) percent inhibition was observed at 300 μM

[e]- 42 ± 4 (n = 2) percent inhibition was observed at 30 μM (the concentration of CMZ after 10-fold dilution)

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. K_I and k_{inact} values for CMZ and human liver microsomal and rCYP2E1 [a]

Enzyme Source	K_I value (μM)	k_{inact} value (min^{-1})	k_{inact}/K_I ratio (μL min^{-1} $nmol^{-1}$)
Pooled human liver microsomes	40 ± 7.0	0.35 ± 0.022	8.8
CYP2E1 Supersomes	41 ± 1.7	0.32 ± 0.048	7.8

[a] - Each value represents the mean ± range of two independent experiments

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

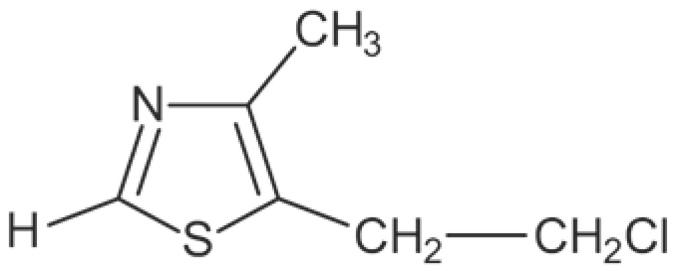


Figure 1

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

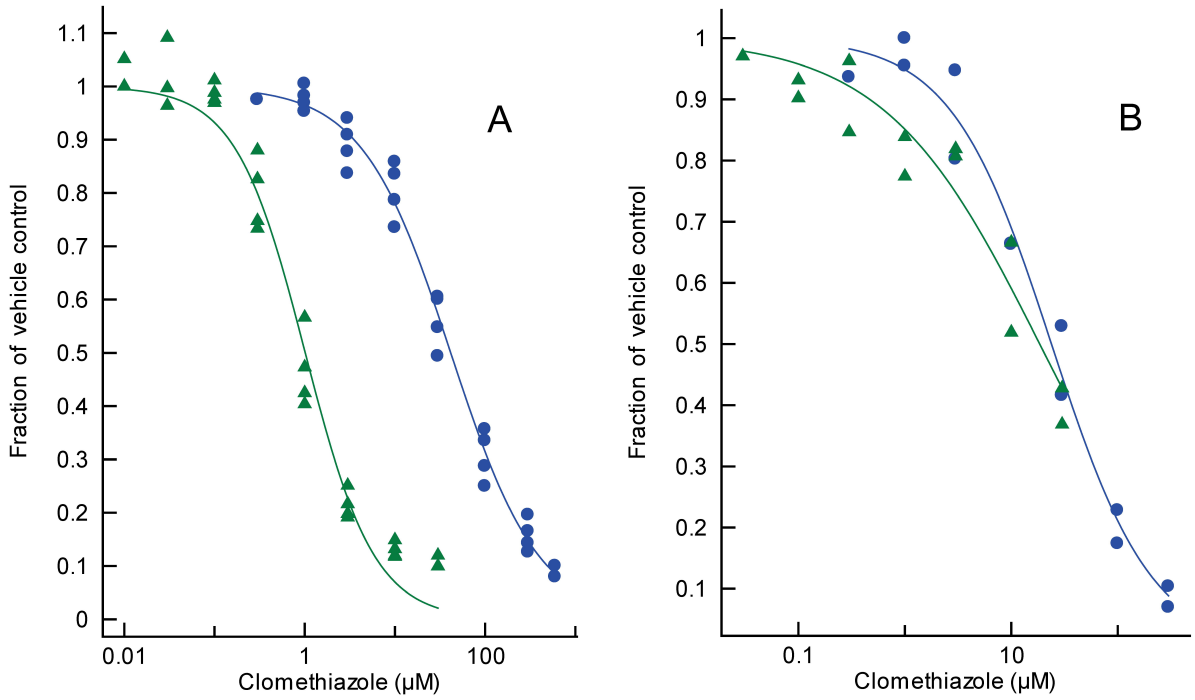


Figure 2

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

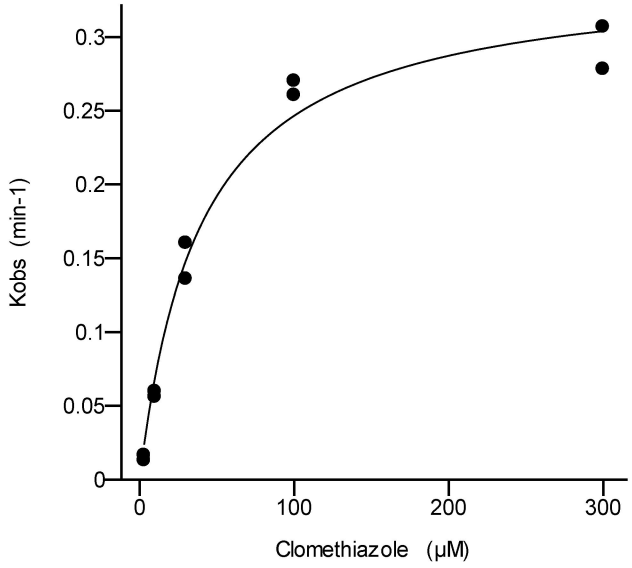


Figure 3

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

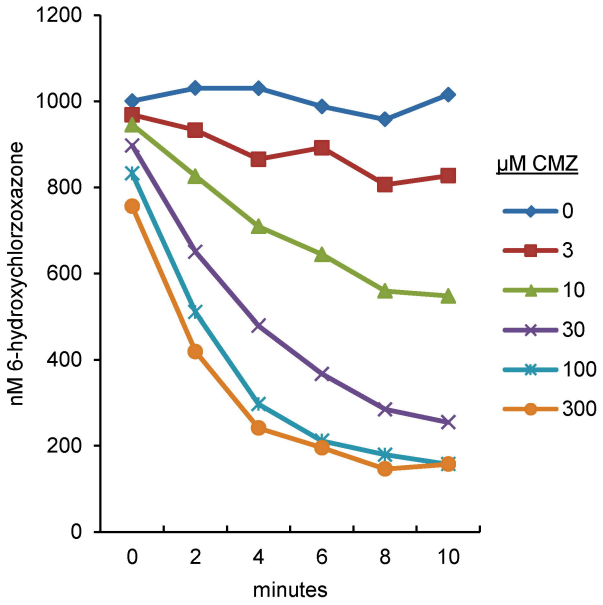


Figure 4

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.

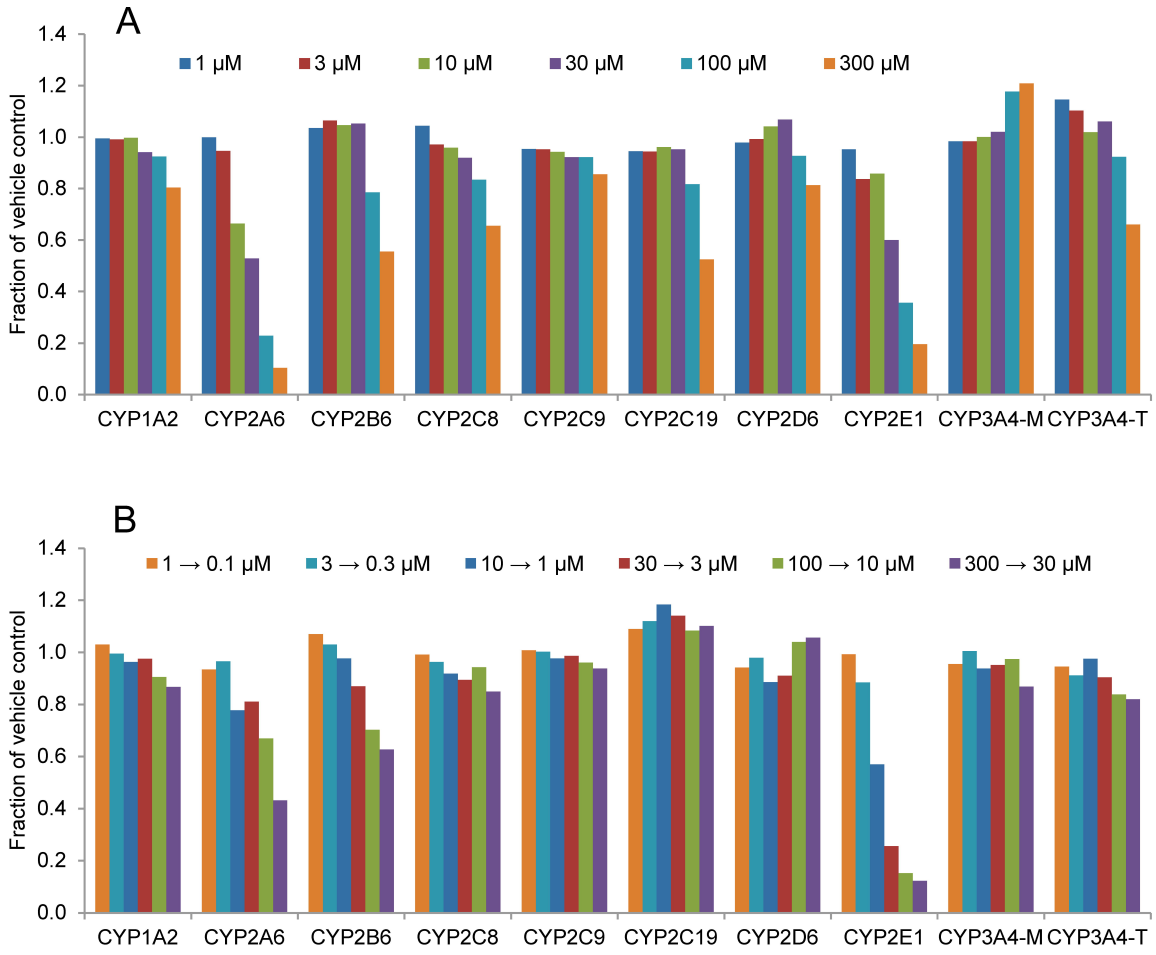
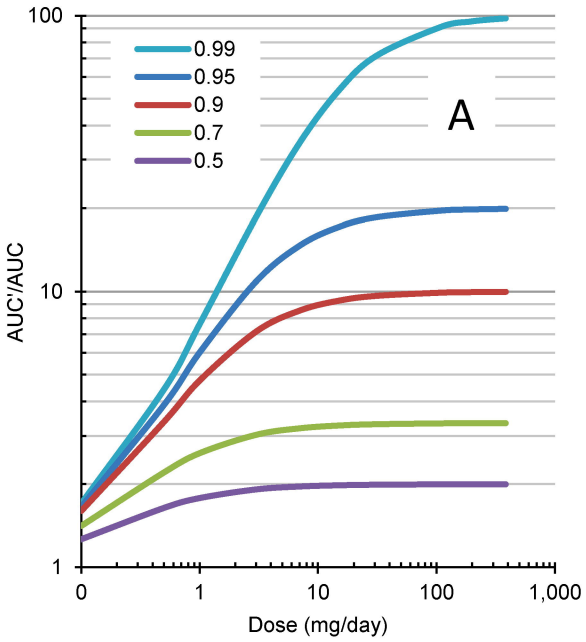


Figure 5

DMD Fast Forward. Published on May 5, 2016 as DOI: 10.1124/dmd.116.070193
This article has not been copyedited and formatted. The final version may differ from this version.



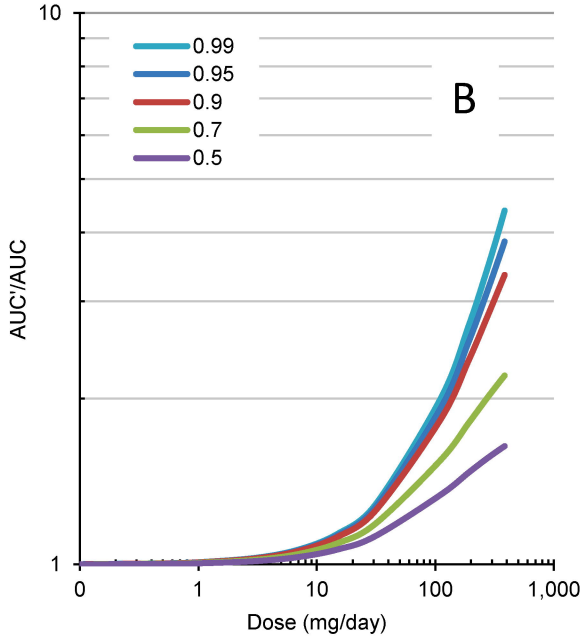


Figure 6

Selective time- and NADPH-dependent inhibition of human CYP2E1 by Clomethiazole

David M. Stresser, Elke S. Perloff, Andrew K. Mason, Andrew P. Blanchard, Shangara S. Dehal, Timothy P. Creegan, Ritu Singh, and Eric T. Gangl

Journal title: Drug Metabolism and Disposition

Supplemental data

Table S1. Summary of terms used in the DDI prediction

Term	Description	Value	Units	Comments
k_{deg}	Degradation rate constant for CYP2E1	0.000232	min^{-1}	Calculated from a mean degradation half-life of 50.5h (range 24.9 – 80h) in 10 subjects estimated from return of chlorzoxazone clearance following a single disulfiram dose (Emery et al, 1999). Although CYP2E1 may degrade in a biphasic manner (Liao et al, 2010), for simplicity, we have assumed a monophasic, first order process.
[I]	$f_{u,b} \times ([I]_{max,b}+F_a \times K_a \times Dose/Q_h)$	75	μM	This is the maximum value calculated using the equation shown and the 384 mg clomethiazole dose. The graph was constructed by modeling a dose range from 0.1 to 384 mg with the assumption that plasma concentrations increased linearly with dose.

$[I]_{max,b}$	Maximum total concentration in blood	8.3	μM	Calculated from mean $[I]_{max,plasma}$ of 1.76 μg/mL or 10.9 μM (Rätz et al, 1999) after oral administration of 600 mg clomethiazole edisylate (equivalent to 384 mg clomethiazole)
k_{inact}	Inactivation rate constant	0.34	min^{-1}	This study
K_I	Concentration that causes half-maximal rate of inactivation	40	μM	This study
K_i	Reversible inhibition	21	μM	This study
$F_{u,\ b}$	Fraction unbound, blood	0.48	dimensionless	Calculated from $F_{u,\ plasma}$/BPP
F_a	Fraction absorbed	1	dimensionless	CMZ is reported to be "well-absorbed"
K_a	Absorption rate constant	0.1	min^{-1}	Value assumed, per FDA guidance
Dose	Oral administered dose	2375500	nmol	Oral administration of 600 mg clomethiazole edisylate (equivalent to 384 mg clomethiazole)
Q_h	Hepatic blood flow	1617	mL/min	FDA guidance (2012)
PPB	Plasma protein binding	63.4 ± 1.6	percent	Data from Jostell et al (1978)
BPP	Blood plasma partition ratio	0.76 ± 0.02	dimensionless	Data from Jostell et al (1978)