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Selective Time- and NADPH-Dependent Inhibition of Human CYP2E1 by Clomethiazole[S]

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ABSTRACT

The sedative clomethiazole (CMZ) has been used in Europe since the mid-1960s to treat insomnia and alcoholism. It has been previously demonstrated in clinical studies to reversibly inhibit human CYP2E1 in vitro and decrease CYP2E1-mediated elimination of chlorzoxazone. We have investigated the selectivity of CMZ inhibition of CYP2E1 in pooled human liver microsomes (HLMs). In a reversible inhibition assay of the major drug-metabolizing cytochrome P450 (P450) isoforms, CYP2A6 and CYP2E1 exhibited IC_{50} values of 24 μM and 42 μM, respectively with all other isoforms exhibiting values >300 μM. When CMZ was preincubated with NADPH and liver microsomal protein for 30 minutes before being combined with probe substrates, however, 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 40 μM and 0.35 minute^{-1}, respectively, and similar to values obtained with recombinant CYP2E1 (41 μM, 0.32 minute^{-1}). The K_I and k_{inact} values, along with other parameters, were used in a mechanistic static model to explain earlier observations of a profound decrease in the rate of chlorzoxazone elimination in volunteers despite the absence of detectable CMZ in blood.

Introduction

Clomethiazole (CMZ; Fig. 1) is a sedative, hypnotic, and anticonvulsant that was introduced into clinical practice in the 1960s and currently is in use for the management of restlessness, agitation, and insomnia in older patients. It may also be used to treat acute alcohol withdrawal (Electronic Medicines Compendium, http://www.medicines.org.uk/emc/medicine/25188).

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 [Gebhardt et al., 1997; Food and Drug Administration (FDA), 2006] the selectivity for inhibition of this enzyme has not been thoroughly investigated or reported.

CYP2E1 is an abundant hepatic cytochrome P450 (P450) isoform known for its role in the metabolism of low-molecular-weight substrates, including chemical carcinogens, organic solvents, and anesthetics (Gonzalez, 2007). Among nonanesthetic 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 (HLMs) and is advantageous over other less selective inhibitors, such as 4-methylpyrazole and diethyldithiocarbamate (Chang et al., 1994; Ono et al., 1996; Newton et al, 1995).

A profound decrease in CYP2E1 catalytic activity, as measured by chlorzoxazone clearance, has been observed after patients received single-dose CMZ (Gebhardt et al., 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 rats (Hu et al., 1994). In light of results

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

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ABBREVIATIONS: AUC, area under the curve; CMZ, clomethiazole; DDI, drug-drug interaction; $F_{m,CYP2E1}$, fraction metabolized by CYP2E1; HLM, human liver microsome; 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; rCYP2E1, recombinant human CYP2E1 (CYP2E1 + OR + b5 supersomes); TDI, time-dependent inhibition.

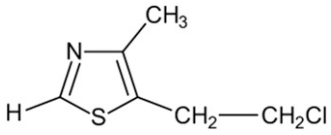

Fig. 1. Structure of CMZ.

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 binds covalently to the heme or apoprotein or formation of a tight-binding inhibitory complex. Such mechanisms would be consistent with those proposed for other thiazole-containing compounds (Obach et al., 2008; Sevrioukova and Poulos, 2010; Subramanian et al., 2010; Rock et al., 2014). 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], 6β-hydroxytestosterone, 6β-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 HLMs (UltraPool HLM 150) pooled from 75 male and 75 female white 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).

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 CMZ (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 glucose-6-phosphate, 0.4 U ml^{-1} glucose 6-phosphate dehydrogenase, 3.3 mM magnesium chloride) in a 400-μl volume. After a 10-minute warmup period, reactions were initiated with the addition of HLM. After the 5-minute incubation, reactions were terminated by the addition of 100 μl of 200 nM 6-hydroxychlorzoxazone $D_2{}^{15}N$ in acetonitrile containing 0.1% formic acid. For K_i experiments, conditions were similar except CMZ 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 time-dependent inhibition (TDI), incubations were conducted with and without a 30-minute preincubation step. With CYP2E1, an additional 10-minute 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 minutes with and without an NADPH-regenerating system. In a final volume of 400 μl, CMZ at varying concentrations [e.g., 0 (water vehicle), 0.1, 0.3, 1.0, 3.0, 10, 30, 100, 300 μM] was preincubated with 10× HLMs (or 5× for CYP2C19 enzyme activity) at 37°C 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 cytochrome-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 minutes, stopped by the addition of 100 μl 0.1% formic acid in acetonitrile containing an internal standard, centrifuged before analysis of metabolites by liquid chromatography-tandem mass spectrometry (Perloff et al., 2009). For CYP2A6, incubations were carried out for 5 minutes and 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 minutes 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 minutes, 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 minutes. Incubations were terminated and processed as described earlier for CYP2E1.

Data Analysis. The IC_{50} values were determined by nonlinear regression using a four-parameter logistic fit model (XLfit, model 205), with maximum and minimum values locked at 100 and 0, respectively. The selection of 0 as the 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, 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 eq. 1:

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]}, \quad (1)$$

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,

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

The raw data at each time point were normalized to the corresponding 0 μM control, and the normalized data were then transformed to the natural log of the percentage of chlorzoxazone 6-hydroxylase activity remaining and the 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 preincubation 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 nonlinear regression using XL-fit (v. 5.3, IDBS, Guildford, UK) software (model 250, with the value of n constrained to 1).

Prediction of Drug-Drug Interaction. The magnitude of a potential drug-drug interaction (DDI) 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 DDIs (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 undetectable in intestine. In this model, shown in eq. 2, 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 DDI:

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

where, in eq. 3, A is the term for enzyme inactivation in the liver:

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

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 in eq. 4 as follows:

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

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 liter/h per 70 kg); The term f_m in eq. 2 refers to the fraction metabolized by CYP2E1.

Results

When CMZ was coincubated with HLM and the probe substrate chlorzoxazone at a concentration of approximately K_m, the IC_{50} value for CYP2E1 was 42 ± 8.9 μM (Fig. 2; Table 1). 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 minutes, before a 10-fold dilution into a secondary incubation containing chlorzoxazone, IC_{50} values shifted lower to 0.96 and 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 $minute^{-1}$, respectively (Fig. 3; Table 2). 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 Fig. 4 without log-transformation of activity to better illustrate the time and concentration dependence of inhibition response that might be expected in a reaction 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 $minute^{-1}$, 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 the direct inhibition response with eight other P450 enzymes (Fig. 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, Fig. 2B, blue), slightly lower than values for CYP2E1. Less potent inhibition was found with other enzymes (Fig. 5, Panel A), 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). When CMZ was preincubated with NADPH and microsomal protein for 30 minutes before being combined with probe substrates, much more potent inhibition was observed for CYP2E1 Table 1, Fig. 5, Panel B). Therefore, CMZ inactivated CYP2E1 (Tables 1 and 2; Fig. 5, Panel A) 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 minutes. Although 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, Figure 2) and does not meet the criteria of a 1.5-fold shift to indicate TDI, as suggested by Grimm et al. (2009).

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 DDI study is required. In this model, the net effect of enzyme induction, reversible inhibition, and time-dependent inhibition is incorporated, both in the liver and gut. We invoked the

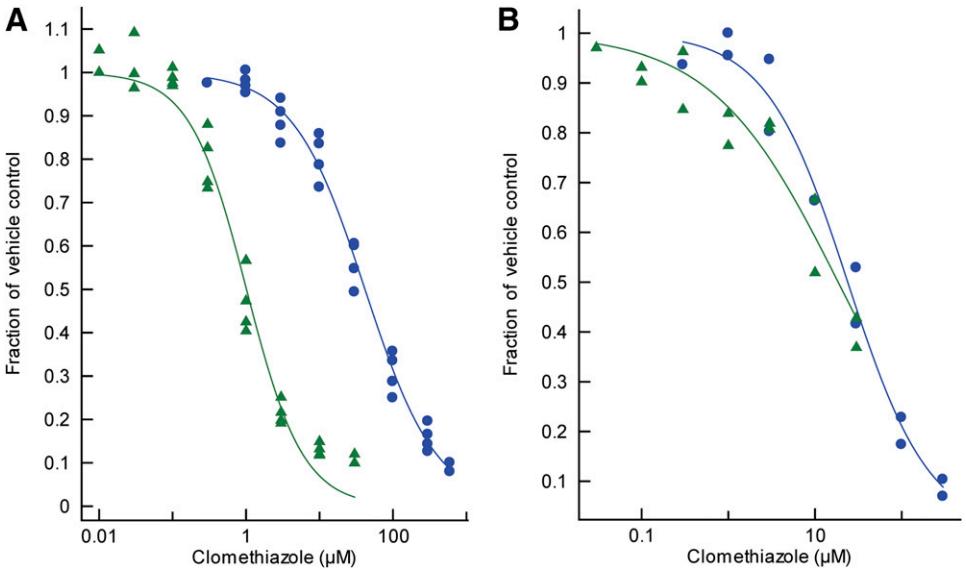

Fig. 2. Effect of preincubation of CMZ with HLM and NADPH on CYP2E1-catalyzed chlorzoxazone 6-hydroxylase activity (A) and CYP2A6-catalyzed coumarin 7-hydroxylase activity (B). Data from four (CYP2E1) or two (CYP2A6) independent experiments were combined to construct the global curve fit for each condition.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

TABLE 1

Summary of clomethiazole (CMZ) inhibition parameters for CYP2E1, CYP2A6, and CYP2B6[a]

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

ND, not done.

[a]Each value represents the mean ± S.D. of 4 (CYP2E1, IC_{50} value with and without 30 minutes of preincubation) or range of two (all other conditions) independent experiments.

[b]Preincubation of CMZ with human liver microsomes (HLMs) and NADPH. Preincubation of CMZ at concentrations up to 100 μM with HLMs in the absence of NADPH for 10 or 30 minutes before the 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]45 ± 0.8 (n = 2) percent inhibition was observed at 300 μM.

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

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 (eq. 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 (Fig. 6A) or both reversible and TDI was examined (Fig. 6B). From this analysis, it is expected that enzyme inactivation by CMZ would have a much greater impact on AUC change than would pure reversible inhibition.

Discussion

Our results confirm previous observations that CMZ is a direct inhibitor of human CYP2E1 when coincubated 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 $\mu l\ min^{-1}\ nmol^{-1}$ (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 cytochromes known to metabolize CMZ (Centerholt et al., 2003).

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 that 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. CYP2A6 was inhibited to a similar extent as CYP2E1; therefore, under conditions of reversible inhibition, CMZ is not ideal as an inhibitor probe of CYP2E1. 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 as a monoselective 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 minutes at a nominal concentration of 30 μM, followed by a 10-fold dilution into an incubation containing the substrate under investigation. A nondilution 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 minutes) would probably be needed to suitably inactivate CYP2E1. With a longer preincubation period, nonspecific and CMZ-independent activity loss of other cytochromes may occur.

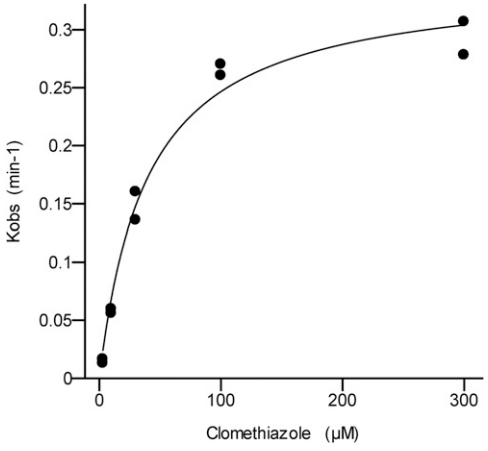

Fig. 3. Determination of K_I and k_{inact} parameters for the inactivation of chlorzoxazone 6-hydroxylation by CMZ in pooled HLMs. Each data point represents the mean of duplicates and data from two independent experiments are shown. The K_I and k_{inact} values obtained with HLMs and rCYP2E1 are shown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

TABLE 2

K_I and k_{inact} values for clomethiazole (CMZ) and human liver microsomal and rCYP2E1[a]

Enzyme Source	K_I Value	k_{inact} Value	k_{inact}/K_I Ratio
Pooled human liver microsomes	μM	min^{-1}	$\mu l\ min^{-1}\ nmol^{-1}$
	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.

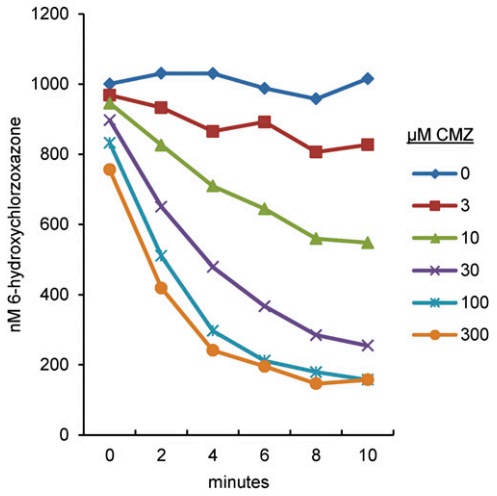

Fig. 4. Loss of chlorzoxazone 6-hydroxylation activity observed after preincubation of CMZ at the concentrations indicated with 1 mg/ml HLM in the presence of an NADPH-regenerating system. The 6-hydroxychlorzoxazone formation was determined after a 10-fold dilution into a secondary incubation containing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

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} values at 4, 2, and 1 mg/ml are 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. CMZ 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 Fig. 2, it is evident that CMZ did not completely inactivate chlorzoxazone 6-hydroxylase activity, even after a 30-minute preincubation with 600 μM CMZ. One explanation for this is that CMZ fails to significantly inhibit the 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 P450 enzymes is a mechanism more prone to elicit DDIs. 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 eq. 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 (Fig. 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 (0.000145–0.000477 minute^{-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 shown in Fig. 6B, 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

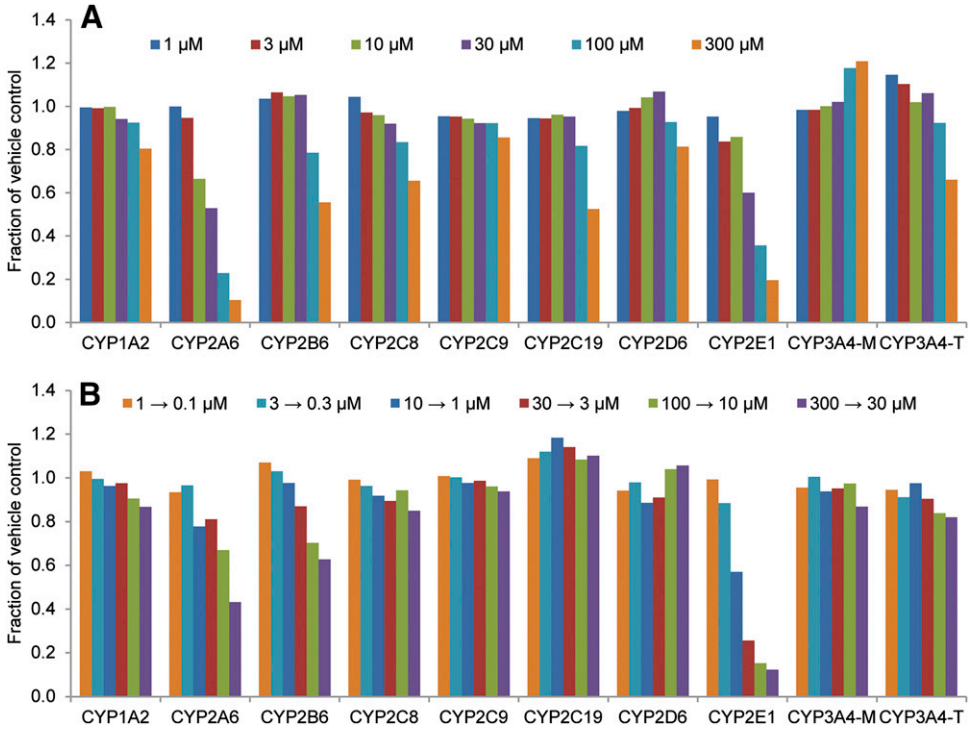

Fig. 5. Effect of CMZ on P450 isoform activity in pooled HLMs. Enzyme activities were conducted as described in the *Materials and Methods* and in Perloff et al. (2009). (A) Direct, reversible inhibition assay. (B) Time-dependent inhibition assay in which CMZ was incubated with HLM and NADPH for 30 minutes before 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.

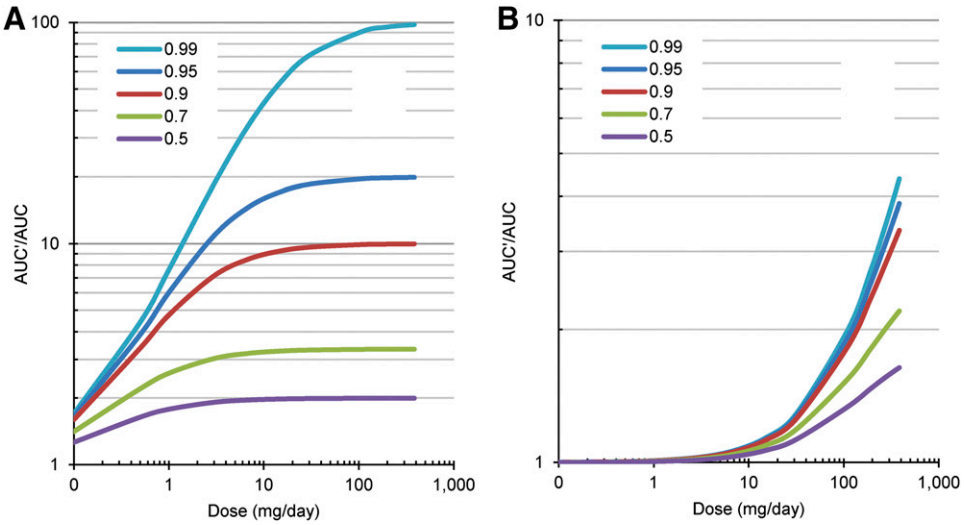

Fig. 6. Simulated effect of CMZ on AUC ratio of plasma concentrations of a CYP2E1 substrate with different levels of $F_{m,CYP2E1}$ deploying a mechanistic static model (eq. 2) incorporating both TDI and reversible inhibition (A) or reversible inhibition only (B); in the latter simulation, the *A* term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

plasma concentrations occur approximately 1 hour after administration, with an elimination half-life of about 4 hours (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 with drug-free volunteers, the AUC of chlorzoxazone, given in a 250-mg dose, increased 3.4-fold in alcoholic patients receiving daily 1.2–2.4 g CMZ at least 1–3 days before 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 therapeutic doses of CMZ (0.6–2.3 g/day) 0.5–12 hours before 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 hours 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 hours for the 192-mg dosed patients and until 60 hours for the 384-mg dosed patients. Additionally, CMZ was not detected in blood at 12 hours postdosing 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; Subramanian et al., 2010; Rock et al., 2014). It has been postulated that initial events leading to metabolic activation of the thiazole and subsequent covalent binding are epoxidation of the C4-5 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 because of 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 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 C4–5 bond discussed above which is sterically hindered by the chloro-ethyl side chain. Together, multiple mechanisms of metabolism-dependent inactivation of CYP2E1 (and CYP2B6) 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 patient monitoring when CMZ is given before drugs that are CYP2E1 substrates.

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Authorship Contributions

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.

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

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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)