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Mechanism-based inactivation of Human Cytochrome P450 2E1 by Diethyldithocarbamate

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Non-Standard Abbreviations: ALDH, aldehyde dehydrogenase; GSH, glutathione; DLPC, dilauroylphosphatidylcholine; DDC, diethyldithiocarbamate; 7-EFC, 7-ethoxy-4-(trifluoromethyl)-coumarin; SDETG, S-(N,N-diethylthiocarbamoyl)glutathione

Abstract

Although disulfiram has been known to inactivate CYP2E1 for more than 20 years, the mechanism has not yet been elucidated. A metabolite of disulfiram, diethyldithocarbamate (DDC), is converted by CYP2E1 to a reactive intermediate that subsequently inactivates the protein leading to mechanism-based inactivation. Mass spectral analysis of the inactivated human 2E1 protein demonstrates that the inactivation is due to the formation of an adduct of the reactive metabolite of DDC with the apoprotein. These data along with mass spectral analysis of a reactive intermediate trapped with glutathione (GSH) indicate the involvement of a reactive intermediate with a molecular mass of 116 Da. Our results suggest that this binding involves formation of a disulfide bond with one of the eight cysteines in CYP2E1. The inactivation of wild type CYP2E1 as well as two of its polymorphic mutants, CYP2E1*2 and CYP2E1*4 were also investigated. For wild type CYP2E1, the K_I was 12.2 μM and the K_{inact} was 0.02 min^{-1}. The K_I values for the two polymorphic mutants were 227.6 and 12.4 μM for CYP2E1.2 and CYP2E1.4, and the k_{inact} values were 0.0061 and 0.0187, respectively. These data indicate that DDC is a much less efficient inactivator of CYP2E1.2 than it is of either the wild type or CYP2E1.4 variant.

Introduction

Disulfiram (Antabuse) has been used therapeutically for more than 60 years for the treatment of alcoholism because of its ability to inhibit aldehyde dehydrogenase (ALDH) (Hald and Jacobsen, 1948; Gaval-Cruz and Weinshenker, 2009). This inhibition leads to a build up of acetaldehyde in those patients who continue to consume alcohol. The elevated levels of acetaldehyde lead to nausea, vomiting, and headaches (Kitson, 1977).

Disulfiram has been shown to have a variety of other therapeutic uses due to its ability to inhibit several other pharmacologically important target enzymes. One of the enzymes inhibited by disulfiram is dopamine β-hydroxylase (DBH), which metabolizes dopamine to norepinephrine (Musacchio et al., 1966). This ability to inhibit the metabolism of dopamine to norepinephrine has resulted in some physicians prescribing disulfiram as a means to promote cocaine abstinence (Gaval-Cruz and Weinshenker, 2009). A third enzyme which is inhibited by disulfiram is glutathione S-transferase, which is involved in the conjugation of endogenously formed electrophilic compounds with GSH (Ploemen et al., 1996).

Disulfiram inhibits these enzymes by two different mechanisms; both of which are dependent on the bioactivation of disulfiram by the body. The first step in these mechanisms involves the reduction of disulfiram in the blood to its monomer, diethyldithocarbamate (DDC), by the glutathione reductase system in

erythrocytes (Cobby et al., 1977). DDC has been shown to be the metabolite of disulfiram responsible for the inhibition of DBH (Goldstein, 1966). Since, the catalytic activity of DBH is copper-dependent, the depletion of copper by DDC causes the loss of DBH activity (Goldstein, 1966). The inactivation of ALDH by disulfiram also is a result of the metabolic conversion of DDC by a series of enzymes to ultimately form methyl N, N-diethylthiocarbamoyl sulfoxide (MeDTC-SO). This compound can then undergo N-dealkylation resulting in the formation of a covalent adduct with ALDH. Formation of this adduct on the $cysteine_{302}$ of ALDH is responsible for the inactivation of the catalytic activity (Lipsky et al., 2001).

Another enzyme that is inhibited by the administration of disulfiram is human cytochrome P450 2E1 (CYP2E1). CYP2E1 is involved in the metabolism of numerous low molecular weight xenobiotics including acetaminophen, benzene, and chlorzoxazone, as well as anesthetics such as halothane (Koop, 1992; Lieber, 1997). Disulfiram has been used for a long time as an inhibitor of CYP2E1 in various in vivo studies (Doroshyenko et al., 2009). It has also been proposed that inhibition by disulfiram could be used to prevent the activation of nitrosamine carcinogens by P450 2E1 (Wattenberg et al., 1989). The inhibition of CYP2E1 by disulfiram has been reported to be due to mechanism-based inactivation (MBI) of the P450 by an oxidized metabolite of disulfiram formed by the 2E1, which reacts with the enzyme leading to inactivation (Guengerich et al., 1991). One of the primary reasons that disulfiram is widely used for inhibition of CYP2E1 in human studies is because of its selectivity. In addition, it is relatively

non-toxic, except when the patient consumes alcohol. Studies have shown that disulfiram has no effect on human P450's 2C9, 2C19, 2D6, or 3A4 (Kharasch et al., 1999).

Although the ability of disulfiram to act as a mechanism-based inactivator of CYP2E1 has been known for 20 years, the exact mechanism by which it causes this inactivation has not been elucidated. We report here that disulfiram by itself does not inactivate CYP2E1 in an in vitro reaction; however, its reduced form, DDC, does inactivate CYP2E1 through the formation of a covalent adduct with CYP2E1. We have identified the metabolite of DDC that is responsible for the inactivation of CYP2E1.We have also characterized the kinetics for the inactivation of the wild type human CYP2E1 as well as two of its commonly occurring polymorphic forms, CYP2E1.2 and CYP2E1.4. These polymorphisms result in the substitution of the arginine residue at position 76 of 2E1 by a histidine and the valine at position 179 by an isoleucine. These two amino acid substitutions have been found in Caucasian and Chinese populations with frequencies estimated to be between 2.4-2.6% (Hu et al., 1997; Fairbrother et al., 1998).

Materials and Methods

Chemicals. NADPH, GSH, catalase, dilauroylphosphatidylcholine (DLPC), disulfiram, and sodium diethyldithiocarbamate (DDC) were purchased from Sigma-Aldrich (St. Louis, MO). 7-Ethoxy-4- (trifluromethyl)-coumarin (7-EFC) was obtained from Invitrogen (Carlsbad, CA). All other chemicals and solvents used were the highest purity available from commercial sources.

Purification of Enzymes. The plasmid for human CYP2E1 was a generous gift from Dr. James R. Halpert (University of California at San Diego, La Jolla, CA). CYP2E1 and the two variants (*2 and *4) were expressed in truncated forms in which the hydrophobic membrane-spanning domain had been removed (Δ3-21), and in addition a His-tag was added onto the C terminus. The CYP2E1 proteins were over-expressed in *Escherichia coli* C41 (DE3) cells and purified to homogeneity as described previously (Scott et al., 2001) Truncated bacterially expressed rabbit CYP2E1 was purified as described previously (Larson et al., 1991; Kent et al., 1998). NADPH-cytochrome P450 reductase and cytochrome b_5 were expressed and purified as described previously (Lin et al., 2005)

Site Directed Mutagenesis of CYP2E1. The mutations of CYP2E1 were accomplished with the QuikChange site-directed mutagenesis kit according to the manufacturer's protocol (Stratgene, CA). The primers used for the mutagenesis were 5'-CGTGGGCTCGCAGCACATGGTGGTGATGCACGG-3' and 5'-CCGTGCATCACCACCATGTGCTGCGAGCCCACG-3' for CYP2E1*2 and 5'-CCAAGGGCACAGTCGTAATACCAACTCTGGACTCTG-3' and 5'-CAGAGTCCAGAGTTGGTATTACGACTGTGCCCTTGG-3' for CYP2E1*4. The

site-specific mutations were confirmed by DNA sequencing at the University of Michigan Sequencing Core facility.

Enzyme Assay and Inactivation of P450s. To assess catalytic activity, CYP2E1 (1.2 nmoles) was reconstituted with reductase and cytochrome b_5 (1:2:2 ratio) with 200 μg/ml DLPC in a final volume of 800 μl for 1 hour on ice. The primary reaction mixtures for the inactivation studies contained 100 pmol CYP2E1 in the reconstituted system and 10 units of catalase in a final volume of 100 μl of 100 mM potassium phosphate buffer, pH 7.3. Different concentrations of disulfiram or DDC were added to separate primary reactions. These reactions were then initiated by the addition of NADPH to a final concentration of 1.3 mM. At the times indicated, aliquots (15 μl) of the primary reaction mixture were removed and transferred to a secondary reaction mixture containing 0.3 mM NADPH, and 0.1 mM 7-EFC in 100 mM potassium phosphate buffer, pH 7.3. The secondary reaction mixtures were incubated for 15 minutes at 28 ^{o}C and then the reactions were terminated by the addition of 100 μl of acetonitrile. The activity remaining was determined by measuring the fluorescent intensity of the product at 520 nm with excitation at 410 nm. Kinetic analyses were performed using Graphpad Prism version 5 for Mac (Graph Pad Software, La Jolla CA; www.graphpad.com).

Analysis of the Primary Metabolite of DDC Formed by 2E1 Using HPLC. CYP2E1 (1.2 nmol) was reconstituted as described above with reductase (2.4 nmol) in a total volume of 800 μl. Reactions were carried out in 100 μl aliquots with 100 μM DDC. In control samples, 2.6 μl of water was added and in the

metabolism samples, 2.6 μl of 50 mM NADPH was added to initiate the reaction. All samples were incubated at 28°C for 1 hour. At the end of hour samples were injected (10 μl) onto a Shimadzu HPLC. The HPLC column was a Zorbax C18 reverse phase column (4.6 X 25 cm; 300 Å; Agilent Technologies, Santa Clara, CA). The solvent system consisted of solvent A (10 mM ammonium formate in water] and solvent B (100% methanol). The column was eluted with 30% B for 15 min and then a linear gradient from 30% to 95% B was used over 30 min at a flow rate of 0.8 ml/min. The elution was analyzed by a model 996 diode-array detector (Millipore Corporation, Billerica, MA).

Analysis of the Modified CYP2E1 Protein by ESI-LC/MS After Inactivation by Sodium Diethyldithiocarbamate. Rabbit CYP2E1 was reconstituted with reductase in a 1:1 ratio as described above. The rabbit CYP2E1 was then incubated for 30 min with 100 μM sodium diethyldithiocarbamate and 1.3 mM NADPH in 100 mM potassium phosphate, pH 7.3. After incubation, 50 μl of the reaction mixture was injected onto a reversed-phase C3 column (2x150 mm, 5 mm)(Agilent Technologies, Santa Clara, CA). CYP2E1 was separated from other reaction components with a binary solvent system consisting of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (solvent B) using the gradient: 30% B for 5 min, linearly increased to 90% B in 20 min, and held at 90% B for 30 min. The flow rate was 0.3 ml/min. The molecular masses of the unmodified and DDC-modified CYP2E1 were determined by deconvolution of the apoprotein charge envelopes using the Bio-works software (ThermoFisher Scientific) (Zhang et al., 2009).

LC-MS/MS Analysis of GSH Conjugate. Human CYP2E1 was reconstituted as described above and incubated with 10 mM GSH, 100 μM DDC, and 1.3 mM NADPH at 28° C for 30 min. The samples were then added to a 1 ml AccuBond ODS-C18 solid phase extraction cartridge (Agilent Technologies, Santa Clara, CA) previously washed with 2 ml of methanol followed by 2 ml of water. After the samples were added to the columns, the cartridges were washed with 2 ml of water and then eluted with 2 ml of methanol followed by 0.3 ml of acetonitrile. The samples eluted in the acetonitrile and methanol were dried under N_2 gas and resuspended in 70 μl of 50% acetonitrile containing 0.5% acetic acid. The samples were analyzed by LC-MS/MS as described previously (Lin et al., 2009).

Results

Inactivation of Human CYP2E1 in the Reconstituted System by Disulfiram and Diethyldithiocarbamate. Both disulfiram and DDC have been known to be inhibitors of CYP2E1 in vivo for a long time. The inactivation kinetics of human CYP2E1 by both disulfiram and DDC were investigated in our in vitro reconstituted system. Human CYP2E1 in the reconstituted system was incubated either with control solvent (DMSO), 20 mM disulfiram, or 200 μM DDC in water. After incubation for 30 minutes, aliquots were transferred to a secondary reaction mixture containing NADPH and EFC. After 15 minutes the reactions were terminated and the fluorescence was measured for each of the reactions as described in Materials and Methods. As shown in Fig.1, the CYP2E1 sample containing disulfiram exhibited no loss in activity when compared to the control; however, the sample containing DDC demonstrated a significant loss of activity (49%) as has been reported previously (Guengerich et al., 1991). These results demonstrate that disulfiram does not inactivate CYP2E1 in the reconstituted system but that DDC is a good inactivator.

Kinetics of the Mechanism-based Inactivation of CYP2E1 by Diethyldithocarbamate. Fig 2 shows the time courses for the inactivation of CYP2E1 at various concentrations of DDC. The inactivation of the 7-EFC O-deethylation activity of CYP2E1 exhibited pseudo-first order kinetics with respect to time and required NADPH. Linear regression analyses of the time course data were used to determine the initial rate constants for inactivation (k_{obs}) at the various concentrations for DDC. From the double reciprocal plot (Fig 2) of the

values of K_{obs} and the concentration of DDC, the K_I was determined to be 12.2 μM and the k_{inact} was 0.02 min^{-1}.

Inactivation of Polymorphic P450 2E1 by DDC. Several genetic polymorphisms of human CYP2E1 have been reported (Hu et al., 1997). We investigated two that have amino acid substitutions in exons 2 and 4, CYP2E1*2 and CYP2E1*4, respectively. These polymorphisms have been estimated to be present in 2.4-2.6% of the Caucasian and Chinese populations (Hu et al., 1997; Fairbrother et al., 1998). Previous studies on the catalytic properties of these proteins have demonstrated that CYP2E1.4 possesses properties similar to those of the wild type CYP2E1.1; however, CYP2E1.2 exhibits kinetic properties significantly different from the wild type and they were substrate dependent (Hanioka et al., 2003).

The inactivations of the two polymorphic CYP2E1 forms were investigated as described above. As shown in Table 1, the K_i values for CYP2E1.2 and CYP2E1.4 are 227.6 and 12.4 μM, respectively, and the k_{inact} values are 0.006 and 0.019 respectively. The values for k_{inact}/K_I for these two proteins are 2.6 x 10^{-6} and1.5 x 10^{-3}, respectively. Thus, the k_{inact}/K_i for CYP2E1.2 was approximately1000 fold less than the wild type whereas CYP2E1.4 exhibited a relatively small change.

HPLC Analysis of the Metabolism of DDC. Since the metabolism of DDC by purified CYP2E1 in the reconstituted system has not previously been reported, we investigated DDC metabolism using HPLC. DDC was incubated with

reconstituted CYP2E1 in the presence and absence of NADPH. Prior to metabolism, DDC exhibited a UV-spectrum with peaks at 244 and 323 (Fig 3A), as reported previously (Ploemen et al., 1996), and it eluted as a single peak with a retention time of 29.5 min (Fig 3B). Incubation of DDC with 2E1 in the reconstituted system with NADPH produced one primary product having a peak at 221.1 in the UV-spectrum (Fig 3C) and which eluted at a slightly earlier time of 27.8 min (Fig 3D).

Covalent Binding of DDC to the CYP2E1 Apoprotein. To determine whether the mechanism-based inactivation of CYP2E1 by DDC is due to the formation of adducts of DDC with the protein or the heme, we performed LC-MS analysis of the inactivated protein. For these studies we incubated rabbit 2E1 with DDC in the reconstituted system. These studies were performed with rabbit 2E1 since it can readily be separated from reductase whereas we have not yet been able to separate human CYP2E1 from reductase. As shown in Fig 4A, the native unmodified CYP2E1 exhibited a single peak with a mass of 53798 in the MS spectrum. This corresponds well with the molecular weight predicted based on the amino acid sequence as well as previously published results (Blobaum et al., 2002). After incubating CYP2E1 with DDC and NADPH for 30 minutes, two mass peaks were observed in the MS spectrum at 53798 (Fig 4A) and 53925 Da (Fig 4B). The former mass is that for the unmodified CYP2E1 and the latter is that for the modified CYP2E1, which exhibited an increase in mass of approximately 115 Da.

The magnitude of this change in the mass of the protein suggested that DDC might be forming a disulfide bond with a cysteine on CYP2E1. In order to test for the presence of a disulfide bond in the protein that could be cleaved by the addition of DTT, we performed the inactivation by DDC as before and then we performed a second incubation for 30 min at 30°C in the presence of 10 mM DTT. We then analyzed the samples by ESI-LC/MS and found that the sample incubated with DTT had lost the adduct and exhibited a mass spectrum similar to that in Fig 4A whereas a sample incubated in the absence of DTT retained the adduct. However, the activity of the protein could not be regained after incubation in the presence of DTT (data not shown).

LC-MS/MS Analysis of a Reactive Metabolite of DDC Formed by CYP2E1. The formation of reactive intermediates during the metabolism of DDC by CYP2E1 in the reconstituted system was investigated by trapping the intermediate(s) with GSH and analyzing the product by LC-MS/MS. LC-MS/MS analysis of the reaction mixture indicated the formation of one metabolite that eluted at approximately 14.3 min with the MH+ at m/z 423 . The proposed structure (Fig 5C) of the adduct is based on the fragmentation patterns shown in Figs 5A and B. In fig. 5A, the fragment at 404 in the MS2 spectrum of the GSH conjugate is due to the loss of oxygen. The fragment at 347 is due to neutral loss of glycine (75 Da). The fragment at m/z 293 is the product of neutral loss of anhydroglutamic acid (129). The fragment at 277 is the product of neutral loss of glutamine (146). The fragments at 218 and 190 are the products from the loss of both glycine and anhydroglutamic acid, and then the subsequent loss of carbon

monoxide (CO). The peak at 149 is the S-(N,N-diethylthiocarbamoyl). The peak at 115 in the MS3 spectrum of the 276 fragment (Fig.5B) is due to the additional loss of the sulfur from the S-(N,N-diethylthiocarbamoyl). The spectrum in Fig 5A is almost identical to that reported by Jin et.al (1994) for SDETG.

Discussion

The use of disulfiram in studies aimed at inactivating CYP2E1 in vivo has been reported recently (Doroshyenko et al., 2009); however the mechanism by which it causes inactivation has not been reported. We report here that disulfiram is not the compound directly responsible for inactivation of CYP2E1, but that its reduced form, diethyldithocarbamate, is responsible for the inactivation (Fig 1). Using purified human CYP2E1 we were able to show that disulfiram does not inactivate P450 at concentrations up to 20 mM; however, DDC caused a significant loss in activity in a time- and concentration-dependent manner in the presence of NADPH. These results indicate that for studies performed in vivo disulfiram must first be reduced in the body to DDC prior to its interaction with CYP2E1 leading to inactivation.

We have also characterized the kinetics for the inactivation of wild type CYP2E1 as well as two of its single amino acid genetic polymorphic forms. The wild type 2E1 exhibits a K_I of 12.2 µM and a k_{inact} of 0.020 min $^{-1}$ (Table 1). The polymorphic CYP2E1.4 demonstrated similar characteristics to the wild type with a K_I of 12.5 µM and a k_{inact} of 0.0187 min $^{-1}$. This was not surprising since previous studies have also shown that this mutation has no affect on enzymatic activity (Hu et al., 1997). The polymorphic CYP2E1.2 had previously been reported to exhibit slower rates of metabolism than the wild type for some substrates (Hu et al., 1997), as well as faster rates for others (Hanioka et al.,

2003). We found that DDC is not a very good inactivator of CYP2E.2 exhibiting a K_I of 227.6 μM and a k_{inact} of 0.0061(Table 1). Thus, in terms of overall efficiency, as measured by K_{inact}/K_I, the inactivation of CYP2E1.2 is a thousand fold less efficient than the inactivation of the wild type 2E1. The CYP2E1.2 polymorphism is a change of residue 76, which is conserved in many mammalian species, from an arginine to a histidine (Song et al., 1986; Khani et al., 1988; Freeman et al., 1992; Komori et al., 1992). The crystal structure of CYP2E1 indicates that Arg^{76} is part of the $\beta_{1\text{-}2}$ structural unit, but that it is oriented towards the solvent (Porubsky et al., 2008). Therefore, it is difficult to explain the mutation's significant impact on the catalytic functionality of CYP2E1; however, possible explanations could include alterations in the substrate entry or product egress channels, effects on the incorporation of the 2E1 into the membrane, or effects on the protein dynamics of the P450. The lack of any significant alteration in the catalytic properties of the polymorphism in CYP2E1.4, in which an isoleucine is substituted for a valine, is probably due to the structural similarities of the two amino acid residues and the fact that position 179 is on the periphery of the protein.

Diode array spectral analysis of the metabolite of DDC formed by 2E1 indicates a change in the UV spectrum from having two peaks at 244 and 323 to a single peak at 221 and the metabolite exhibits a change in the elution time on HPLC from 29.5 min to 27.7 min (Fig 3). No other peaks were observed in the elution profile for the metabolism of DDC by CYP2E1, which indicates that either

this product was the only one produced by 2E1 or that other products could not be detected spectrally.

To determine the mass of the apoprotein adduct formed by the metabolism of DDC to a reactive intermediate, we incubated rabbit CYP2E1 with DDC and then analyzed the modified protein by electrospray ionization liquid chromatography mass spectrometry. Our results demonstrated that the loss in the catalytic activity of CYP2E1 is due to the formation of a single adduct of DDC with the protein. The evidence for this comes from the increase in the observed mass of the inactivated CYP2E1 apoprotein of 115 mass units. (Fig 4B), In addition, HPLC analysis of the inactivated protein reaction mixture showed no change in the unmodified heme content or the presence of any modified heme (data not shown).

To determine a more accurate measurement of the adduct we performed a GSH trapping experiment. Data from the CYP2E1 protein adduct as well as products observed by Jin et al.(1994) helped to narrow down the mass range to search. Jin et al. (1994) administered disulfiram to mice and used mass spectrometry to examine GSH conjugates in the bile. They observed five DDC metabolites and the second most prevalent product S-(N,N-diethylthiocarbamoyl)glutathione (SDETG) exhibited a MH+ ion at m/z 423. This would correspond to a DDC metabolite of 116 Da, which is within the error for the mass of the adduct we observed with the apoprotein. Analysis of the products formed following incubation of DDC with reconstituted human CYP2E1 and GSH indicated the formation of a GSH adduct with an ion at m/z 423. Analysis of the

fragmentation pattern indicated peaks that corresponded to the characteristic loss of glycine, anhydroglutamic acid, and glutamine.

We postulate that this metabolite could inactivate CYP2E1 by forming a disulfide bond with one of the 8 cysteines that are present in the apoprotein. The loss of the DDC-adduct and reversion of the mass of the 2E1 to that of the unmodified protein in the presence of DTT provides addition evidence in support of this hypothesis. We are currently attempting to determine which of the 8 cysteine residues in CYP2E1 is covalently modified by the metabolite of DDC leading to the inactivation. Attempts to digest the modified protein, separate it by LC, and sequence it by MS/MS have so far not been successful, presumably due to the lability of the disulfide bond. Studies involving site-specific mutagenesis of the cysteines are in progress.

References

Blobaum AL, Kent UM, Alworth WL and Hollenberg PF (2002) Mechanism-based inactivation of cytochromes P450 2E1 and 2E1 T303A by tert-butyl acetylenes: characterization of reactive intermediate adducts to the heme and apoprotein. *Chem Res Toxicol* **15:**1561-1571.

Cobby J, Mayersohn M and Selliah S (1977) The rapid reduction of disulfiram in blood and plasma. *J Pharmacol Exp Ther* **202:**724-731.

Doroshyenko O, Fuhr U, Kunz D, Frank D, Kinzig M, Jetter A, Reith Y, Lazar A, Taubert D, Kirchheiner J, Baum M, Eisenbrand G, Berger FI, Bertow D, Berkessel A, Sorgel F, Schomig E and Tomalik-Scharte D (2009) In vivo role of cytochrome P450 2E1 and glutathione-S-transferase activity for acrylamide toxicokinetics in humans. *Cancer Epidemiol Biomarkers Prev* **18:**433-443.

Fairbrother KS, Grove J, de Waziers I, Steimel DT, Day CP, Crespi CL and Daly AK (1998) Detection and characterization of novel polymorphisms in the CYP2E1 gene. *Pharmacogenetics* **8:**543-552.

Freeman JE, Stirling D, Russell AL and Wolf CR (1992) cDNA sequence, deduced amino acid sequence, predicted gene structure and chemical regulation of mouse Cyp2e1. *Biochem J* **281 (Pt 3):**689-695.

Gaval-Cruz M and Weinshenker D (2009) mechanisms of disulfiram-induced cocaine abstinence: antabuse and cocaine relapse. *Mol Interv* **9:**175-187.

Goldstein M (1966) Inhibition of norepinephrine biosynthesis at the dopamine-beta-hydroxylation stage. *Pharmacol Rev* **18:**77-82.

Guengerich FP, Kim DH and Iwasaki M (1991) Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4:**168-179.

Hald J and Jacobsen E (1948) A drug sensitizing the organism to ethyl alcohol. *Lancet* **2:**1001-1004.

Hanioka N, Tanaka-Kagawa T, Miyata Y, Matsushima E, Makino Y, Ohno A, Yoda R, Jinno H and Ando M (2003) Functional characterization of three human cytochrome p450 2E1 variants with amino acid substitutions. *Xenobiotica* **33:**575-586.

Hu Y, Oscarson M, Johansson I, Yue QY, Dahl ML, Tabone M, Arinco S, Albano E and Ingelman-Sundberg M (1997) Genetic polymorphism of human CYP2E1: characterization of two variant alleles. *Mol Pharmacol* **51:**370-376.

Jin L, Davis MR, Hu P and Baillie TA (1994) Identification of novel glutathione conjugates of disulfiram and diethyldithiocarbamate in rat bile by liquid chromatography-tandem mass spectrometry. Evidence for metabolic activation of disulfiram in vivo. *Chem Res Toxicol* **7:**526-533.

Kent UM, Roberts ES, Chun J, Hodge K, Juncaj J and Hollenberg PF (1998) Inactivation of cytochrome P450 2E1 by tert-butylisothiocyanate. *Chem Res Toxicol* **11:**1154-1161.

Khani SC, Porter TD, Fujita VS and Coon MJ (1988) Organization and differential expression of two highly similar genes in the rabbit alcohol-inducible cytochrome P-450 subfamily. *J Biol Chem* **263:**7170-7175.

Kharasch ED, Hankins DC, Jubert C, Thummel KE and Taraday JK (1999) Lack of single-dose disulfiram effects on cytochrome P-450 2C9, 2C19, 2D6, and 3A4 activities: evidence for specificity toward P-450 2E1. *Drug Metab Dispos* **27:**717-723.

Kitson TM (1977) The disulfiram--ethanol reaction: a review. *J Stud Alcohol* **38:**96-113.

Komori M, Kikuchi O, Sakuma T, Funaki J, Kitada M and Kamataki T (1992) Molecular cloning of monkey liver cytochrome P-450 cDNAs: similarity of the primary sequences to human cytochromes P-450. *Biochim Biophys Acta* **1171:**141-146.

Koop DR (1992) Oxidative and reductive metabolism by cytochrome P450 2E1. *FASEB J* **6:**724-730.

Larson JR, Coon MJ and Porter TD (1991) Alcohol-inducible cytochrome P-450IIE1 lacking the hydrophobic NH2-terminal segment retains catalytic activity and is membrane-bound when expressed in Escherichia coli. *J Biol Chem* **266:**7321-7324.

Lieber CS (1997) Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev* **77:**517-544.

Lin HL, Kent UM and Hollenberg PF (2005) The grapefruit juice effect is not limited to cytochrome P450 (P450) 3A4: evidence for bergamottin-dependent inactivation, heme destruction, and covalent binding to protein in P450s 2B6 and 3A5. *J Pharmacol Exp Ther* **313:**154-164.

Lin HL, Zhang H and Hollenberg PF (2009) Metabolic activation of mifepristone [RU486; 17beta-hydroxy-11beta-(4-dimethylaminophenyl)-17alpha-(1-propynyl)-estra-4 ,9-dien-3-one] by mammalian cytochromes P450 and the mechanism-based inactivation of human CYP2B6. *J Pharmacol Exp Ther* **329:**26-37.

Lipsky JJ, Shen ML and Naylor S (2001) In vivo inhibition of aldehyde dehydrogenase by disulfiram. *Chem Biol Interact* **130-132:**93-102.

Musacchio JM, Goldstein M, Anagnoste B, Poch G and Kopin IJ (1966) Inhibition of dopamine-beta-hydroxylase by disulfiram in vivo. *J Pharmacol Exp Ther* **152:**56-61.

Ploemen JP, van Iersel ML, Wormhoudt LW, Commandeur JN, Vermeulen NP and van Bladeren PJ (1996) In vitro inhibition of rat and human glutathione S-transferase isoenzymes by disulfiram and diethyldithiocarbamate. *Biochem Pharmacol* **52:**197-204.

Porubsky PR, Meneely KM and Scott EE (2008) Structures of human cytochrome P-450 2E1. Insights into the binding of inhibitors and both small molecular weight and fatty acid substrates. *J Biol Chem* **283:**33698-33707.

Scott EE, Spatzenegger M and Halpert JR (2001) A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function. *Arch Biochem Biophys* **395:**57-68.

Song BJ, Gelboin HV, Park SS, Yang CS and Gonzalez FJ (1986) Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P-450s. Transcriptional and post-transcriptional regulation of the rat enzyme. *J Biol Chem* **261:**16689-16697.

Wattenberg LW, Sparnins VL and Barany G (1989) Inhibition of N-nitrosodiethylamine carcinogenesis in mice by naturally occurring organosulfur compounds and monoterpenes. *Cancer Res* **49:**2689-2692.

Zhang H, Lin HL, Walker VJ, Hamdane D and Hollenberg PF (2009) tert-Butylphenylacetylene is a potent mechanism-based inactivator of cytochrome P450 2B4: inhibition of cytochrome P450 catalysis by steric hindrance. *Mol Pharmacol* **76:**1011-1018.

Footnotes

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B. Please send reprint requests to: Paul F. Hollenberg, PhD, Department of Pharmacology, The University of Michigan, 1150 W. Medical Center Dr. 2301 MSRB III, Ann Arbor, MI 48109-5632. Email: phollen@umich.edu

Figure Legends

Figure 1. Effects of disulfiram and diethyldithocarbamate on human CYP2E1 in the reconstituted system. CYP2E1 was reconstituted with reductase in primary reaction mixtures containing either 20 mM disulfiram or 200 μM DDC. Samples were incubated for 0 or 30 minutes following the addition of NADPH and then transferred to secondary reaction mixtures containing 0.1 mM EFC and 0.3 mM NADPH. The secondary reactions were incubated for 15 minutes, terminated by the addition of 100 μl acetonitrile and the HFC fluorescence was measured as described in Materials and Methods. Percentages are calculated based on the amount of signal for the 30 min incubations divided by the signal for the 0 min incubations from experiments done in triplicate on two separate days.

Figure 2. Time and concentration-dependent inactivation of CYP2E1 by DDC. Reconstituted CYP2E1 was incubated with 0 (●), 5(■), 10 (▲), 20 (▼) 40 (◆), 200 (○), and 400 μM (□) DDC. Aliquots were removed at the times indicated and assayed for residual activity as described in Materials and Methods. The insert shows the double reciprocal plot for the initial rates of inactivation as a function of the concentrations of DDC. The kinetic constants were determined from the double reciprocal plot. The data shown represent the average of four separate experiments done in duplicate.

Figure 3. Metabolism of diethyldithocarbamate by CYP2E1. DDC (100 μM) was incubated with reconstituted CYP2E1 (1pmol/μl) in the reconstituted system. The control and NADPH+ samples were incubated for 30 minutes at 28 ^{o}C.

Samples were then injected onto the HPLC, eluted, and monitored as described in Materials and Methods. The control samples (A) exhibited two peaks in the UV-spectrum absorbing at 244 and 323 nm and eluted as a single peak having an elution time at 29.5 min (B). The sample incubated with NADPH (C) gave a single major metabolite with a UV-spectrum peak at 221.1 (C) and having an elution time of 27.8 min (D).

Figure 4. Deconvoluted mass spectra for the native CYP2E1 (A) and the inactivated CYP2E1 (B) after reaction with diethyldithocarbamate under turnover conditions. The inactivations were performed as described in Materials and Methods. Aliquots of the primary reaction mixture containing 50 pmoles of CYP2E1 were loaded onto a C4 column and the CYP2E1 samples were separated from the rest of the components in the reconstituted mixture as described in Materials and Methods. The molecular masses of the CYP2E1 samples were analyzed by ESI-LC/MS as described in Materials and Methods.

Figure 5. LC-MS/MS analysis of the DDC-GSH conjugate formed during metabolism by reconstituted CYP2E1. (A) The MS2 spectrum of the DDC-GSH conjugate eluting at 14.34 min with an m/z of 423 is shown. (B) The MS3 from the 276 fragment. (C) The proposed structure of the DDC-GSH conjugate is inset into the MS/MS spectrum and the dashed lines are the sites of fragmentation as described in the text.

Table 1. Inactivation Kinetics for the CYP2E1 Alleles.

2E1 allele	K_I (μM)	k_{inact}	k_{inact}/K_I
human *1	12.2	0.0202	1.6×10^{-3}
human *2	228	0.0061	2.7×10^{-6}
human *4	12.5	0.0187	1.5×10^{-3}
rabbit	5.8	0.0075	1.3×10^{-3}

* P450 (15 pmol) was incubated with DDC in the reconstituted system and the kinetic values were calculated as described in Materials and Methods.

Figure 1

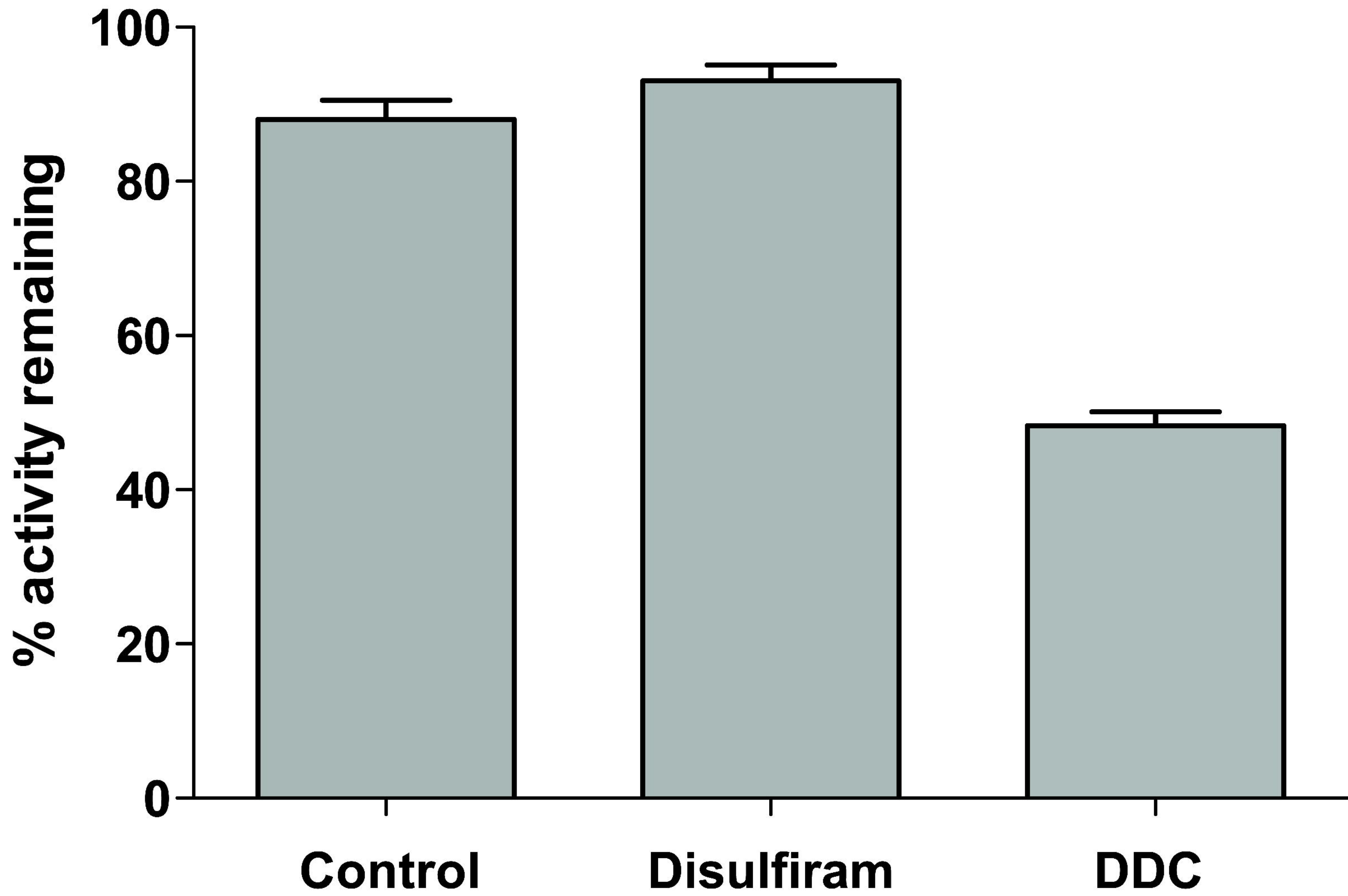


Figure 2

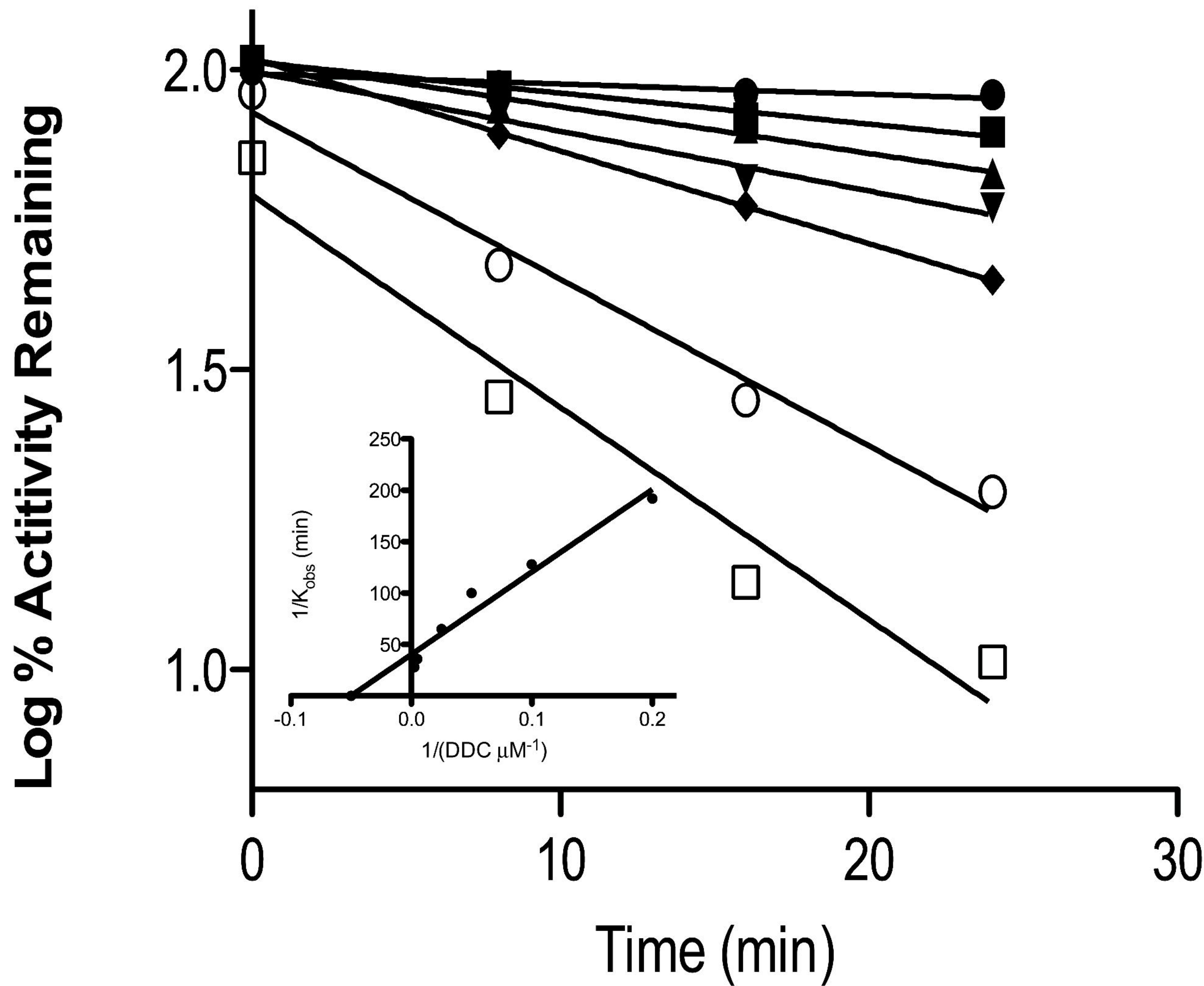


Figure 3

A

53798

Relative Abundance

0 10 20 30 40 50 60 70 80 90 100

53000 53200 53400 53600 53800 54000 54200 54400

mass

B

53915

53800

Relative Abundance

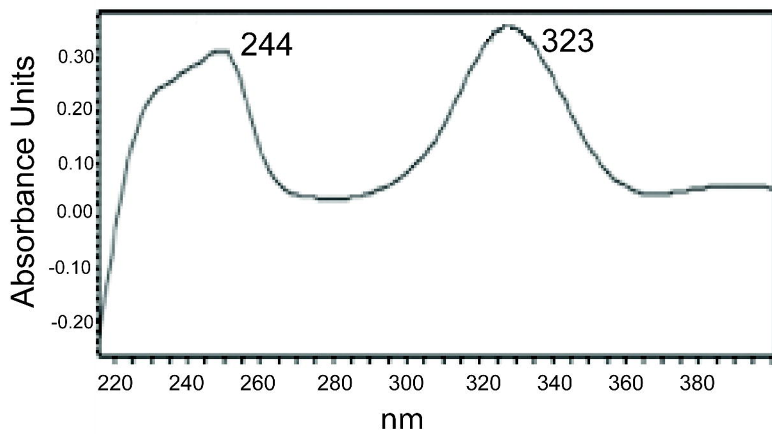
0 10 20 30 40 50 60 70 80 90 100

53000 53200 53400 53600 53800 54000 54200 54400

mass

Figure 4

A



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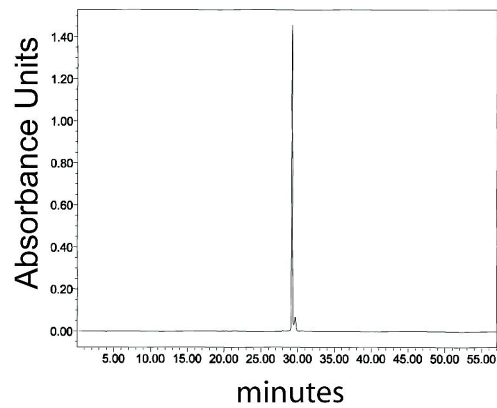

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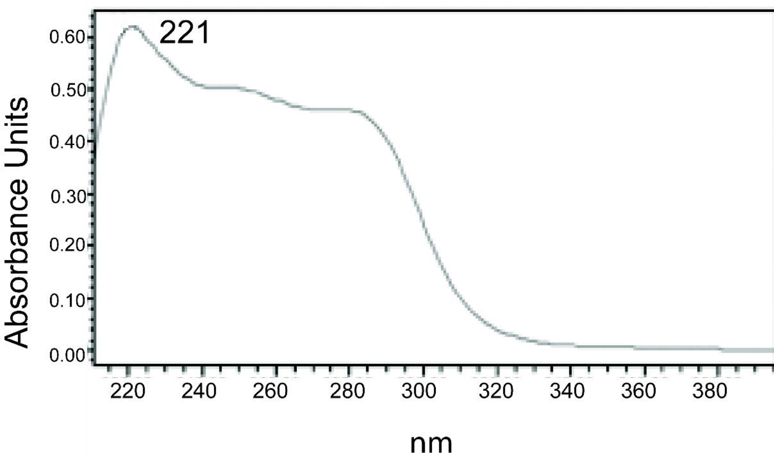


D

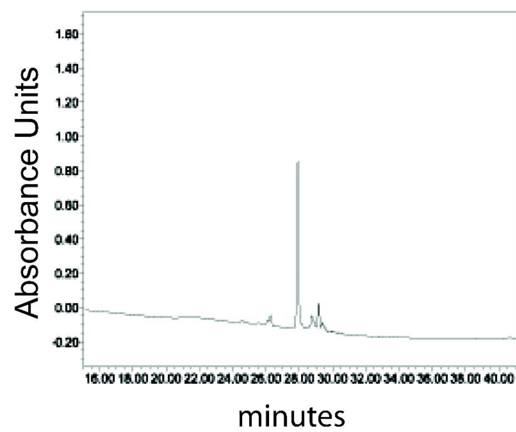
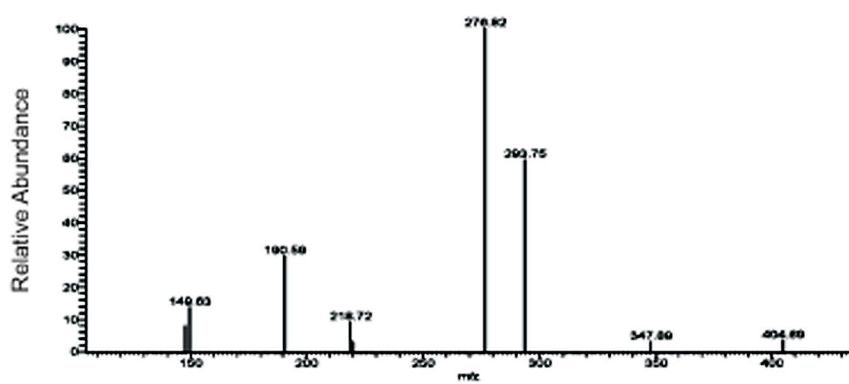
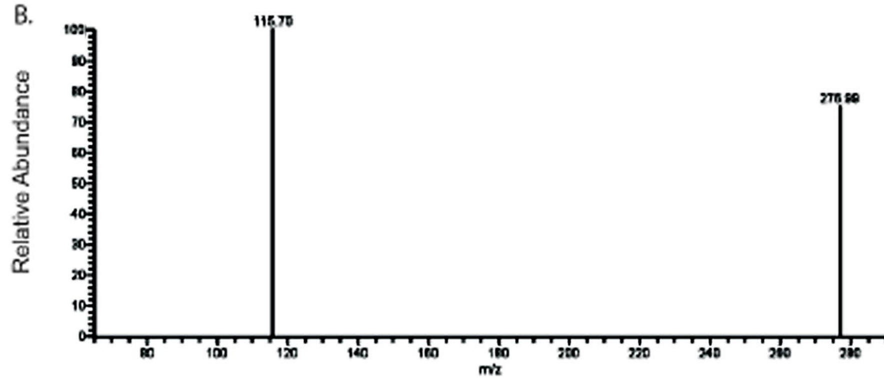


Figure 5

A.



B.



C.