

## Research

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# Interactions between Over-the-Counter and Illicit Drugs Utilizing Cytochrome P450 Metabolism: Potential for Exacerbation of Pharmacological Response

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## ABSTRACT

**Aim:** To determine the interaction of over-the-counter (OTC) and illicit psychostimulants at the cytochrome P450 enzyme, CYP2D6. CYP2D6 is responsible for 20% of hepatic Phase I metabolism and is a site of drug-drug interactions, leading to increased drug toxicity.

**Materials and Methods:** We examined the effects the OTC drugs; 1) the prototype H<sub>2</sub>-antagonist cimetidine (CMT) and 2) the opioid agonist cough suppressant dextromethorphan (DEX); as well as two scheduled drugs, methamphetamine (MA) and 3,4-methylenedioxymethamphetamine (MDMA) for their ability to interfere with CYP2D6 activity. Assays with human CYP2D6 determined the inhibitory potential (IC<sub>50</sub>) of each drug. Kinetic analysis (V<sub>max</sub> and K<sub>m</sub>) was accomplished using rodent hepatic microsomes.

**Results:** Maximum inhibition of CYP2D6 activity following exposure to CMT+MDMA was significantly reduced 75-85% compared to quinidine (control) values. These data showed inhibitory effects in CYP2D6 activity in each compound tested. Alterations in CYP2D6 activity may result in complex drug-drug interactions leading to elevated plasma levels of drugs and increased risk for toxicity. Assays using rat CYP2D2 demonstrated V<sub>max</sub> elevations in the CMT group (493%) compared to control (naïve, no treatment) values (19.9±5.1 pmol/mg protein/min). The K<sub>m</sub> was increased 218% in CMT compared to controls (3.1±0.5 μM). Collectively, all MA challenged groups exhibited increases in total enzyme [V<sub>max</sub>; 280-490%] and affinity [K<sub>m</sub>; 165-220%] values compared to the control group. The increase in both V<sub>max</sub> and K<sub>m</sub> suggests that the low affinity/high capacity CYP2D2 isoform is upregulated.

**Conclusion:** Our findings suggest that *in vivo*, MA acts as a CYP2D2-inducer, which will lead to altered secondary drug metabolism, increasing the risk of drug-related toxicity. Coupled with the ability of CMT and DEX to interfere with MA metabolism, a complex drug-drug interaction is possible, leading to increased toxicity. Our findings substantiate the hypothesis that the combination of illicit and OTC drugs could result in complex drug-drug interactions increasing the risk for severe drug-related toxicity.

**KEY WORDS:** Cimetidine; Methamphetamine; Dextromethorphan; Ecstasy; CYP2D6; Hepatosomes; Methylenedioxymethamphetamine.

**ABBREVIATIONS:** MA: Methamphetamine; AM: Amphetamine; OTC: Over-the-counter; MDMA: 3,4-Methylenedioxymethamphetamine; CMT: Cimetidine; DEX: Dextromethorphan; CYP: Cytochrome P450; NE: Norepinephrine; DA: Dopamine; 5-HT: Serotonin; MDA: 3,4-Methylenedioxymethamphetamine; AMMC: 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin; HHMA: 3,4-Dihydroxymethamphetamine; AHMC: 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin; PCP: Phencyclidine; DXO: Dextrophan.

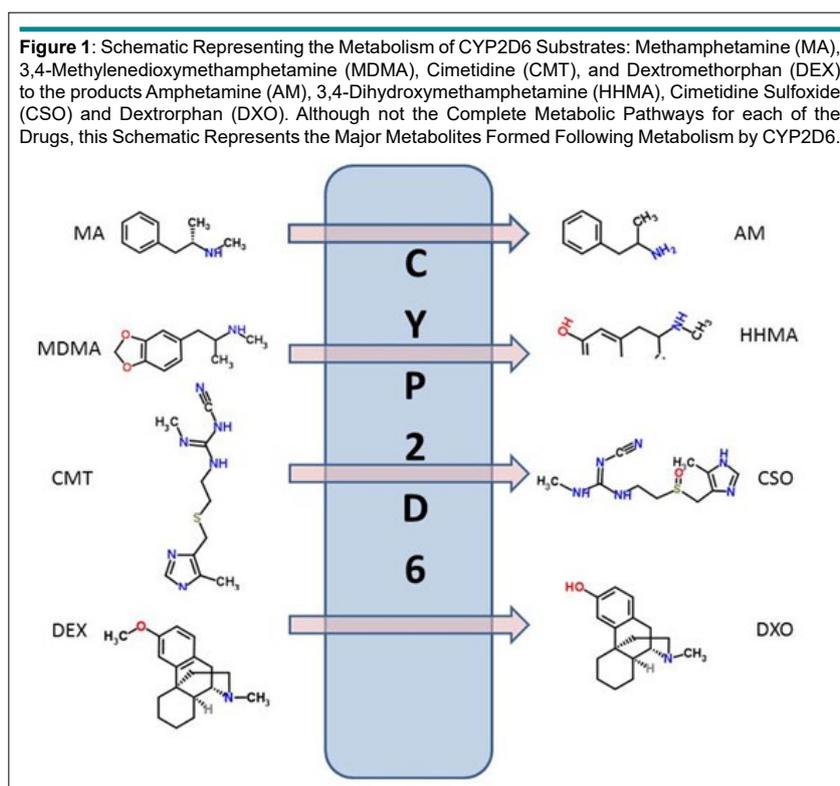
## INTRODUCTION

The cytochrome P450 (CYP) enzyme system is a superfamily of hemoproteins that catalyze the Phase I metabolism of numerous xenobiotics and is usually associated with hepatic metabolism.<sup>1,2</sup> CYP enzymes are also found in the small intestine, kidney, lung, and brain.<sup>3</sup> CYP2D6 is the second largest drug metabolizer (20%) and is an important part of the phase I drug metabolism, which transforms a functional group or adds a functional group to the drug to introduce or unmask polar bodies.<sup>4,5</sup> The active catalytic site of CYP2D6 contains acidic amino acid residues (Asp301 and Glu216) and as such will bind substrates which contain basic nitrogen and planar aromatic ring groups.<sup>6</sup> CYP2D6 has been studied extensively due to its genetic polymorphisms and its large number of substrates.<sup>1</sup> Based on genetic profiling, CYP2D6 polymorphisms are separated into the following classifications: 1) poor, 2) normal, and 3) extensive metabolizer. Allelic variants that are prevalent in ethnic populations that are known to be poor metabolizers are the CYP2D6\*10 and CYP2D6\*17 variants.<sup>7</sup> Ethnic populations which express these alleles, would have reduced function and be 'poor' metabolizers, whereas individuals with the 'normal' alleles will be able to adequately metabolize, and the last group, the 'extensive' metabolizers, will either express more of the active alleles, or will have been 'induced' by a secondary drug leading to a greater number of enzyme molecules.<sup>2,8,9</sup> Most drugs or chemicals exhibit reversible inhibition where there is competition for the catalytic site.<sup>9</sup> Inhibition magnitude is a function of the inhibiting agent concentration and the affinity of the agent for CYP2D6.<sup>2</sup> It is clear that the combination of ethnic/genetic effects coupled with the induction/inhibition ability of various drugs, the potential for

drug-drug interactions resulting in an increased incidence of toxicity is significant. Improved understanding of CYP2D6 activity in the presence of illicit drugs will lead to better interpretation and predictions of drug-drug interactions.

Methamphetamine (MA) and 3,4-methylenedioxy-methamphetamine (MDMA) are abused psychostimulant drugs often ingested as part of polydrug use. Drug-drug interactions at the level of the hepatic CYP microsomal system (Phase I reactions) can result in unexpected and dangerous side effects. Interactions between drugs may also affect forensic/medical toxicological analysis and interpretation. OTC-related inhibition of MA or MDMA metabolism will result in the accumulation of the illicit drug leading to increased toxicity. Cimetidine (CMT) and MDMA<sup>10-12</sup> are inhibitors of CYP2D6 whereas dextromethorphan (DEX) and MA are substrates for the CYP2D6 isozyme.<sup>2,13,14</sup> Comparing the actions of CMT and DEX at CYP2D6, CMT exhibits 20-fold lower potency at inhibiting compared to DEX (200  $\mu$ M vs. 10  $\mu$ M).<sup>15</sup> Inhibitors of CYP activity lead to increased drug adverse effects associated with the increase in the drug concentration.<sup>1,2,9</sup> Clemens et al confirmed the increase in adverse effects when MDMA and MA are administered concurrently.<sup>16</sup> The ability of X-ray crystallography to determine the CYP2D6 three-dimensional structure has aided significantly in understanding the mechanism of substrate activity at the CYP2D6 catalytic site.<sup>17</sup> A sample schematic for the metabolism of MA, MDMA, CMT, and DEX (Figure 1) includes the primary metabolite for each of the parent compounds.

Amphetamine (AMP) was first synthesized as a nasal decongestant to replace ephedrine.<sup>18</sup> Later, AMP was used to treat



narcolepsy and hyperactivity, but its abuse skyrocketed.<sup>19</sup> After World War II, AMP and related drugs like methamphetamine (MA) were available without a prescription thus increasing use and abuse. It was not until the Controlled Substances Act (1986) that the regulation of AMP/MA increased and these agents were classified as “C-II” which means that there are medical uses, but the agents are highly addictive.<sup>19</sup> MA is a derivative of AMP and belongs to the phenethylamine class of psychostimulants.<sup>20</sup> MA is commonly accepted as more addictive and favored by drug addicts due to its increased ability to enter the central nervous system (CNS).<sup>19,21</sup> Of the two racemic forms of MA, the *d*-isomer exhibits greater stimulatory potency.<sup>22</sup> The mechanism associated with AMP and MA addiction involves increased activity of the dopaminergic system where dopamine (DA) release is increased, or DA is displaced from its storage vesicles elevating synaptic DA concentration.<sup>19,20</sup> At higher doses, MA reduced both DA and serotonin (5-HT) synthesis.<sup>19,20</sup> MA itself has low potency for stimulating DA release, but is metabolized to *p*-hydroxymethamphetamine (~15%) and the active metabolite AMP (4-7%) *via* CYP2D6. AMP is subsequently metabolized to *p*-hydroxyamphetamine and other inactive metabolites. Currently, MA is an easily obtainable drug, and the ease of production, increased potency, and availability has led to increased MA abuse.

Methylenedioxymethamphetamine (MDMA) was first synthesized as an appetite suppressant, yet never received approval for medical use. It was abused in the 1960s and 1970s for its psychoactive and hallucinogenic properties, and it was classified as “C-I” (no medicinal use, highly addictive).<sup>19</sup> Currently, MDMA is an illicit recreational drug that is very popular at all-night parties or raves. MDMA is an analog of MA formed by methylenedioxy substitution<sup>22</sup> and is structurally similar to mescaline and methylenedioxyamphetamine, MDA.<sup>23</sup> MDMA affects several neurotransmitter systems including norepinephrine (NE), DA, 5-HT, and the neurotransmitter  $\gamma$ -amino butyric acid, GABA.<sup>22</sup> MDMA is a strong and selective 5-HT neurotoxin in numerous animal species, including non-human primates.<sup>24</sup> In rats, MDMA stimulates 5-HT release which is believed to be *via* the reversal of the 5-HT transporter.<sup>22</sup> There are two forms of MDMA: the R(-)-isomer and the more potent neurotoxin, the S(+)-isomer. MDMA is O-demethylated to 3,4-dihydroxymethamphetamine [HHMA] *via* CYP2D6<sup>23,25</sup> followed by N-demethylation to the active metabolite MDA *via* CYP1A2 and CYP2D6.<sup>22</sup> MDA potently inhibits the activity of CYP2D6 through irreversible, covalent, binding to the CYP2D6 catalytic site.<sup>10</sup> When taken orally, MDMA is well absorbed with a half-life of 7 hours in humans, with MDA reaching peak plasma concentrations in 4-6 hours.<sup>26</sup> Nearly two-thirds of MDMA is excreted in the urine unchanged.<sup>22,26</sup> There is evidence to support long-term electrophysiological abnormalities in MDMA users and suggest that typical recreational doses of MDMA are enough to cause long-term altered cortical activity in humans.<sup>24</sup> MDMA and MA, when taken concurrently, can produce greater adverse effects, dependent on the order of administration. The sequence of administration of MA and MDMA appears to have pharmacological relevance.<sup>16</sup> Collectively, these reports suggest that there

could be a synergistic or additive effect of MDMA and MA that may be due to interactions at a common metabolic point, such as CYP2D6.

Cimetidine (CMT) is a commonly used over-the-counter medication for the treatment of acid reflux disease and heartburn. CMT was one of first in the class of histamine H<sub>2</sub> blockers/antagonists that prevent H<sup>+</sup> secretion from the parietal cells into the stomach lumen.<sup>27</sup> CMT can bind to the cytochrome P450 heme iron reactive site, inhibiting all cytochrome-dependent phase I enzyme activity.<sup>28</sup> Due to this interaction at P450 sites, CMT has been associated with many drug-drug interactions involving the inhibition of CYP2D6 and other P450 isozymes.<sup>29,31</sup> When CMT is combined with MA, levels of both MA and AMP were significantly higher in the rat CNS compared to rats that did not receive CMT.<sup>32</sup> CMT is metabolized by P450 enzymes to its major metabolite, an S-oxide.<sup>27,29,33</sup> The half-life of CMT is relatively short (2-4 hours) and is quickly cleared from the body by urinary excretion (70% of unchanged CMT) or the S-oxide form (20%).<sup>34,35</sup> The use of CMT as an acid-reducer is widespread, but the exact mechanism of P450 inhibition is not entirely understood. More work is needed to enhance our understanding of the potential of drug-drug interactions associated with CMT use.

Dextromethorphan (DEX) is an antitussive used in cold and cough medications to relieve non-productive coughs.<sup>36</sup> The abuse of DEX has been recognized since 1975, yet DEX has not been placed on the Controlled Substances Act.<sup>37</sup> DEX does not bind to opioid receptors like classical opioids and it has no analgesic activities; however, it does bind to a site associated with sigma-site ligands and also to the phencyclidine (PCP) N-methyl-D-aspartate (NMDA) glutamate receptor channel site.<sup>38,39</sup> At higher concentrations, recreational users reach dissociative effects similar to PCP or ketamine.<sup>38,40</sup> DEX is a substrate for CYP2D6 and may also interfere with the metabolism of many other drugs like CMT. DEX had been used to determine if a person is a rapid, normal or slow CYP2D6 metabolizer by measuring the rate which the active metabolite dextrorphan (DXO) is formed.<sup>41</sup> Both DEX and DXO have been shown to reduce MA self-administration at doses <30 mg/kg in rats,<sup>42</sup> suggesting that co-administration of DEX and MA may reduce the reinforcing properties associated with MA administration. Quinidine, a prototype substrate for CYP2D6, inhibits the DEXO-demethylation to DXO, thus increasing the DEX/DXO ratio.<sup>36,43-45</sup> After oral administration, DEX undergoes hepatic metabolism (85%) to increase elimination in the urine, but almost 15% of DEX is excreted unchanged.<sup>44,46</sup>

This study examines the interactions between two OTC drugs and two illicit drugs at CYP2D6 and asks: *can OTC drugs interfere with the metabolism of the illicit drugs leading to increased toxicity?* Drugs and Human Performance Fact sheets from the National Highway Traffic Safety Administration<sup>47</sup> also report “potential inhibitors of the CYP2D6 isozyme could decrease the rate of methamphetamine elimination if administered concurrently, while potential inducers could increase the rate of

elimination.” Collectively, this work will improve our understanding of potential drug-drug interactions between OTC and illicit drugs and the potential hazards associated with polydrug use/abuse. This work is significant since unforeseen drug interactions may lead to the misinterpretation of toxicology results.

## EXPERIMENTAL DESIGN

### Chemicals and Drugs

Methamphetamine HCl, 3, 4-methylenedioxymethamphetamine HCl, cimetidine, dextromethorphan-HBr, acetonitrile, dimethyl sulfoxide (DMSO), 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin (AMMC)<sup>48</sup> were purchased from Sigma Chemical Company (St. Louis, MO, USA). The inhibitor screening kit CYP2D6/AMMC was purchased from BD Biosciences (Woburn, MA). The P450 HTS kit include: CYP2D6 (CYP2D6\*1 + P450 reductase), non-fluorescent substrate (AMMC), fluorescent metabolite (AHMC), cofactors (1.3 mM NADP<sup>+</sup>, 66 mM MgCl<sub>2</sub>, and 66 mM glucose 6-phosphate), glucose 6-phosphate dehydrogenase (40 Units/ml in 5 mM sodium citrate buffer, pH 7.5), reaction buffer solution (0.5 M potassium phosphate, pH 7.4, filter sterilized), stop solution reagent (0.5 M Tris base reagent, filter sterilized), CYP2D6 positive control inhibitor (quinidine),<sup>28</sup> and NADPH regenerating system. The ability of this kit to perform *in vitro* screening of CYP2D6 substrates and inhibitors and its comparison to other screening modalities have been previously described.<sup>49,50</sup>

### Animals

Male Sprague-Dawley rats (6-months old/375-425 grams, Harlan Sprague-Dawley Laboratories, Indianapolis, IN) were randomly assigned to treatment groups; group housed, and allowed access to food (Pro-lab Rat, Mouse, and Hamster Chow #3000) and water ad libitum in a temperature controlled room (23±2°C) and 12-hour light cycle. All animals experienced the same environmental conditions over the course of the experiment. Twenty rats (N=4 for each group) were placed in quarantine upon arrival for one week and then moved to the animal colony and allowed to habituate in their environment for at least one week prior to use. Animals were maintained according to NIH guidelines in our USDA-certified facilities. The protocol for the use of animals in this research was approved by the IACUC of Oklahoma State University Center for Health Sciences.

### Inhibition Studies Purified CYP2D6

AMMC, 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin, is a non-fluorescent substrate that is demethylated to the fluorescent metabolite AHMC, 3-[2-(N, N-diethylamino) ethyl]-7-hydroxy-4-methylcoumarin hydrochloride, which was used to screen for interactions with CYP2D6.<sup>51,52</sup> Quinidine, a potent inhibitor of CYP2D6, was used as the control compound to determine intra- and inter- assay

variability. Stock solutions (1 mM) of CMT, DEX, MA, and MDMA were each prepared in HPLC-grade acetonitrile before each CYP2D6 assay. Groups were divided by treatment: CMT, DEX, MA, MDMA, CMT+MA, CMT+MDMA, DEX+MA, DEX+MDMA, CMT+DEX+MA, and CMT+DEX+MDMA. Each drug was serially diluted (1:3) from highest concentrations (20 µM) to lowest concentrations (3 nM) and pre-incubated at 37 °C for 10 min in a black/clear bottom 96-well plates. Separate vehicle assays were performed (maximum 2% acetonitrile) and there was no effect on CYP2D6 activity (data not shown), which is similar to reports by the manufacturer. The enzyme-substrate mix [CYP2D6\*1-P450 reductase + 10 mM AMMC] was added and plates were incubated at 37 °C for 30 min. The conversion of AMMC to AHMC was terminated by the addition of stop reagent and the amount of fluorescent product, AHMC, was determined using a fluorescent plate reader (Synergy HT with KC4 software; Bio-TEK Instruments, Inc., Winooski, VT, USA). Measurement parameters for determining AHMC fluorescence utilized an excitation/emission filter of 360 nm/460 nm. Background values were subtracted from the treatment wells before statistical analysis.

### Inhibition Studies: Rat CYP2D2

**Animals and treatment:** Rats were randomly assigned to one of the 5 treatment groups: The first group was control (naïve); treatment groups included: vehicle control (VC; 0.9% saline and DMSO, 4%); CMT (10 mg/kg); DEX (10 mg/kg); and the combination of CMT+DEX (10 mg/kg each). Rats received a single daily injection from day 1 till day 7 at the same time each day. All compounds were administered *via* intraperitoneal (IP) injections to minimize first-pass effects. On Day 8, the drug-treated rats (not naïve rats) were challenged with a 5 mg/kg IP injection of MA. Seven hours after MA injection, rats were lightly anesthetized using carbon dioxide gas, and sacrificed by decapitation. The median and the left lateral hepatic lobes were harvested and frozen in liquid nitrogen. Samples were stored at -80 °C until use.

**Hepatic microsome preparation:** Hepatic microsomal fractions were prepared with slight modifications as previously described.<sup>53</sup> Briefly, frozen (-80 °C) rat livers were thawed and minced in 2-4 mL of homogenizing buffer (0.1 M potassium phosphate, pH 7.4 and 0.25 M sucrose), then brought to 30 mL with additional homogenizing buffer. Homogenization was completed with 10 strokes at 900 rpm using a Teflon pestle/glass homogenizer (Wheaton, USA). Nuclei and mitochondria were removed by centrifugation at 9,000 xg for 20 min at 4 °C. The resulting supernatant (S1) was centrifuged at 100,000 xg for 60 min. The pellet (P2; containing microsomes) was resuspended in 20 mL of incubation buffer (0.1 M potassium phosphate, pH 7.4, 0.25M sucrose, 1 mM EDTA, and 5% glycerol) and used immediately or stored frozen (-80 °C) until use. Florence et al showed washed microsomes could be stored at -80°C for up to 30 days without loss of activity.<sup>54</sup> All stored microsomes in the present studies were used before the end of the 30 day period.

**Protein Analysis:** Commercially available Coomassie-blue-based protein assay (Bio-Rad, Richmond, CA, USA) based on the Bradford method was used to determine total protein concentration.<sup>55</sup> Based on specific CYP2D2 activity, the calculations for pmol/mgprotein/min was determined.

**In vivo Kinetic Studies:** The *in vivo* kinetic studies used the HTS kit from the *in vitro* studies with modifications. The assay was used to quantify the CYP2D2 (rat) enzyme activity for each of the drug treatment groups following MA challenge by measuring the production of fluorescent AHMC. The activity of CYP2D2 was determined following seven-day exposure to CMT, DEX, and CMT/ DEX, or saline using AMMC as a probe. This probe has been shown to be highly selective for rat CYP2D2, as well as the human CYP2D6 isoform.<sup>48,56</sup> Rat hepatic microsomes were used in place of the purified human CYP2D6 utilized in the *in vitro* assay.

Assays were performed as described above. The enzyme mix for the treatment groups was prepared for each microsomal fraction by adding H<sub>2</sub>O, buffer (0.5 M potassium phosphate, pH 7.4, filter sterilized), and enzyme (microsomal fraction), a 79:20:0.75 mix. For the treatment groups, enzyme mix and AMMC (0.5 μM to 3.29 μM) were mixed in the well for a total volume of 100 μL. Varying AMMC concentrations produced a concentration response curve that determined enzyme kinetics. The plate was incubated for 30 min at 37 °C, and the reaction was terminated by the addition of stop reagent. The fluorescence generated by AMMC was quantified as described previously at excitation/emission wavelengths of 360 nm/460 nm. Assays were performed as four assays (N=4) in duplicate.

### Statistical Analysis

For the *in vitro* CYP2D6 inhibition assays, the inhibitory potency of quinidine and each test compound was determined by measuring the IC<sub>50</sub> value for each compound. This data was curve fit using an iterative nonlinear curve fitting program in PRISM 7.01 (GraphPad Software Inc., San Diego, CA, USA). IC<sub>50</sub> values and the maximum inhibition percentage were then analyzed using the Kruskal-Wallis nonparametric test followed by the Dunn's comparison (using quinidine as the comparison group). A significance level was set at α=0.05.

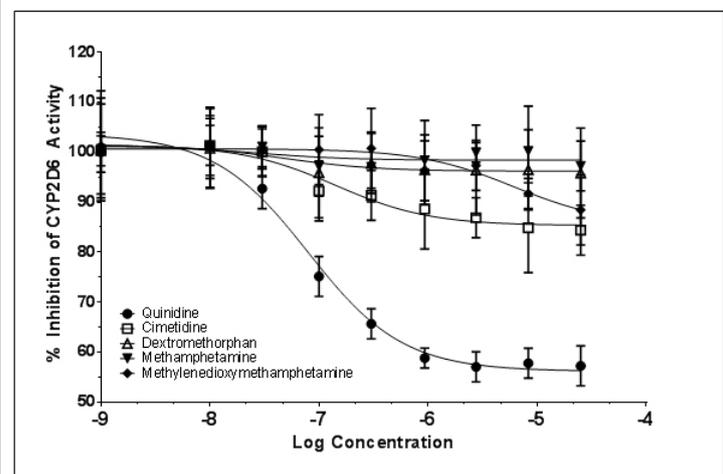
For the *in vivo* enzyme kinetic assay, data was collected similar to above and calculated using a nonlinear fit (rectangular hyperbola) of the data, yielding V<sub>max</sub> and K<sub>m</sub> values. Kinetic data were then analyzed using one-way ANOVA followed by Dunnett's posthoc test to compare each of the treatment groups to the K<sub>m</sub> (μM) and V<sub>max</sub> (pmol of AMHC formed/mg protein/minute) values obtained from naïve rats. The significance level again was set at α=0.05. All data are expressed as the mean±standard error of the mean (SEM).

## RESULTS AND DISCUSSION

### Inhibition Studies Purified CYP2D6

To determine inhibition of CYP2D6 by quinidine and test compounds the IC<sub>50</sub> values were calculated for each inhibition curve (Figure 2). Then, the mean log IC<sub>50</sub> values for each were group were obtained and compared (Figure 3A and 4A). The IC<sub>50</sub> values for each test compound were then compared to the

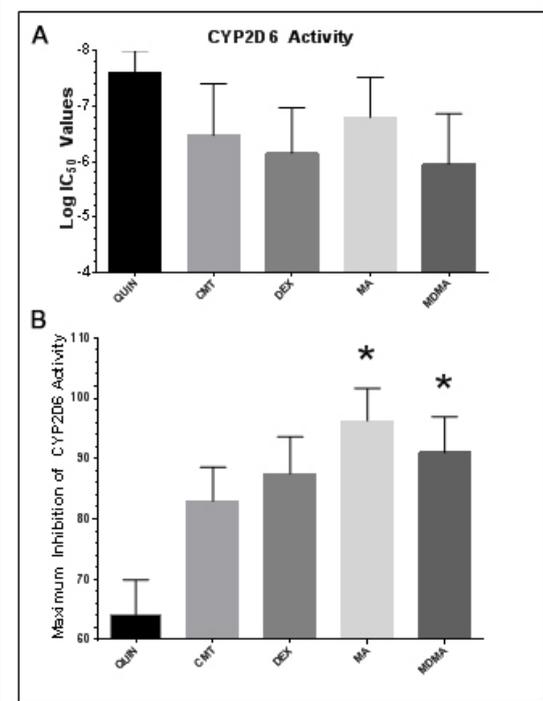
**Figure 2:** Inhibition of CYP2D6 Activity by OTC and Illicit Drugs. Purified Human CYP2D6 was Incubated in the Presence of the Non-Fluorescent 3-[2-(N, N-Diethyl-N-Methylamino) Ethyl]-7-Methoxy-4-Methylcoumarin (AMMC, 10 mM) which was then Demethylated to the Fluorescent Metabolite 3-[2-(N, N-Diethylamino) Ethyl]-7-Hydroxy-4-Methylcoumarin Hydrochloride (AHMC). Enzyme-Substrate Mixtures were incubated with 8 Concentrations of CMT, DEX, MA or MDMA (0.2 nM – 100 μM) for 10 min at 37°C. The Quantity of Fluorescent Product (AHMC) was then Measured with a Fluorescence Excitation/Emission Filter of 360 nm/460 nm. Data was Fit using Nonlinear Regression Analysis and both 1-site and 2-site Models were Compared. Curves were best Fit to the Simpler, 1-site Model and from this Analysis, the IC<sub>50</sub> and Maximum Inhibition Values could be Determined (expressed in Figures 3 and 4). Each of the Data Points Represent 4 Assays (N=4) Performed in Duplicate, Except for the DEX Group which was an N=3. Data then Expressed as the Mean±SEM.



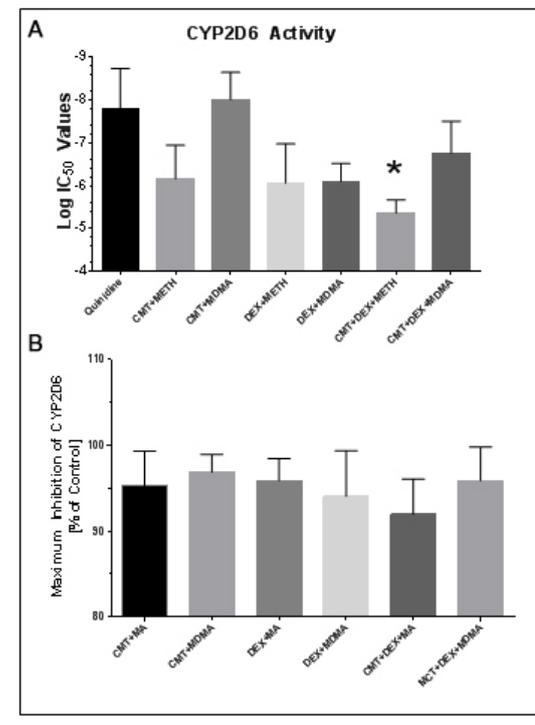
quinidine IC<sub>50</sub> value (3.8 nM) which was used as the ‘control.’ Our values are similar to the values reported elsewhere and in the manufacturer literature (3-11 nM).<sup>50</sup> Figure 2 represents the inhibition curve from each test compound (and quinidine) and the data are expressed as the mean±SEM of 4 assays performed in duplicate for each test compound except for the DEX and CMT+MA groups which are three assays performed in duplicate. Another indicator of effect at CYP2D6 was the measurement of the maximum inhibition elicited by each of the test compounds. The maximum inhibition (35-40%) of quinidine was set as 100% inhibition, and each group was then calculated as a percentage of the quinidine maximum (Figure 3B and 4B). When examining single compound, there was no effect on the log IC<sub>50</sub> values compared to quinidine (H<sub>5,20</sub>=5.66; *p*=0.2263, Figure 3A). Comparing the maximum inhibition of the single test compounds to quinidine revealed a significant effect of treatment (H<sub>5,20</sub>=13.69; *p*=0.0084, Figure 3B) with both the MA and MDMA groups exhibiting significantly less inhibition compared to the quinidine group (*p*<0.05). When

examining the effects of multiple drug exposures, to determine if there may be synergistic, additive or potentiating effects, there was a generalized lowering of log IC<sub>50</sub> values (H<sub>7,21</sub>=14.42; *p*=0.0252, Figure 4A). Comparison to quinidine values revealed that only the CMT+DEX+METH log IC<sub>50</sub> value was significantly (*p*<0.05) reduced compared to quinidine values. In the combination treatment groups, all groups exhibited significantly reduced inhibition compared to quinidine, yet there were no differences between any of the combination treatment groups (Figure 4B). Collectively these data suggest none of the drugs resulted in the inhibition of CYP2D6 to the same extent as quinidine, the prototype CYP2D6 inhibitor. Although, not potent inhibitors, each of the test compounds did elicit some level of inhibition of CYP2D6 activity. Each test compound (single or combination) displayed a lower affinity for CYP2D6 compared to quinidine (usually 10- to 100-fold lower IC<sub>50</sub> values compared to the 3-11nM IC<sub>50</sub> value reported for quinidine). In general, the *in vitro* assays suggest that each of the OTC and illicit drugs can inhibit CYP2D6 to a relatively small extent and that the weak

**Figure 3:** Effects of Single Drug Exposure on CYP2D6 Activity. The IC<sub>50</sub> (A) and Maximum Inhibition (B) Values were Determined from the Nonlinear Analysis (Figure 2). The Values for Quinidine (IC<sub>50</sub> and Maximum Inhibition) were used as the Positive Control to which the other Drug Exposure Effects were Compared. When Examining Single Compound Comparisons (A), there was no Effect (H<sub>5,20</sub>=5.66; *p*=0.2263) on the log IC<sub>50</sub> Values Compared to Quinidine Values. Comparing the Maximum Inhibition (B) of the Single Test Compounds to the Maximum CYP2D6 Inhibition by Quinidine, there was a Significant Effect of Treatment (H<sub>5,20</sub>=13.69; *p*=0.0084) with both the MA and MDMA Groups Exhibiting Significantly Less Inhibition Compared to the Quinidine Group (*p*<0.05). Data are Expressed as mean±SEM of 4 Assays (3 for DEX) Run in Duplicate. \**p*<0.05 Compared to Quinidine Value.



**Figure 4:** Effects of Combination Drug Exposure on CYP2D6 Activity. The IC<sub>50</sub> (A) and Maximum Inhibition (B) Values were Determined from the Nonlinear Analysis (Figure 2). The Values for Quinidine (IC<sub>50</sub> and Maximum Inhibition) were used as the Positive Control to which the other Drug Exposure Effects were Compared. Examining the IC<sub>50</sub> Values (A), the Combination Drug Exposure had Generally Lower IC<sub>50</sub> Values Compared to the Quinidine Groups (H<sub>7,21</sub>=14.42; *p*=0.0252). Comparison to Quinidine Values Revealed that only the CMT+DEX+METH Combination Group had Significantly (*p*<0.05) Lower log IC<sub>50</sub> Values Compared to the Quinidine Group. In the Combination Treatment Groups, all Groups Exhibited Significantly Reduced Inhibition Compared to Quinidine (Approximately 35% Inhibition to 5-10%), yet there were no differences between any of the Combination Treatment Groups (B). Data are Expressed as mean±SEM of 4 Assays (3 for CMT+MA) Performed in Duplicate. \**p*<0.05 Compared to Quinidine Value.



inhibition observed does not exhibit any other characteristics in the combination groups such as synergism, potentiation or additivity.

Each of the drugs tested have reported action at CYP2D6 either as an inhibitor, an inducer, or a substrate. CMT has been reported to have inhibitory effects on CYP2D6 in both humans and rodents.<sup>33,57-59</sup> DEX interacts with a greater number of P450 isozymes, with CYP2D6 being a major contributor to DEX metabolism by O-demethylation.<sup>60</sup> It is clear that OTC drugs such as CMT and DEX can play a significant role in the development of drug-drug interactions leading to increased toxicity by interference with CYP2D6 metabolism. There is an extensive body of work regarding the metabolism of MA and MDMA. CYP2D6 is involved in multiple steps in the metabolism of both MA and MDMA.<sup>61</sup> Earlier reports suggest that the parent compound for MA and its parent compound, amphetamine, act at CYP2D6 with low micromolar affinity.<sup>62</sup> Our findings demonstrate that a potential interaction between OTC and illicit drugs does exist<sup>63</sup> and that the interaction may lead to increased risk of toxicity. Overall, our findings *in vitro* show the calculated  $IC_{50}$  values for the test compounds were reduced by a 75-85% decrease compared to  $IC_{50}$  values associated with quinidine. The  $IC_{50}$  value for quinidine of 3.8 nM was consistent with the value reported by the manufacturer and other researchers.<sup>50,64,65</sup> The efficacy of inhibition or maximum inhibition was lower than the inhibition that was observed with quinidine, suggesting that the test compound only weakly interacted with CYP2D6 compared to quinidine.

Studies using MA and MDMA employed a variety of different methodologies to measure kinetic parameters.<sup>10-12,62,64</sup> Taavitsainen et al reported an  $IC_{50}$  value for MA of 414  $\mu$ M, but this study used DEX as the substrate probe, not AMMC.<sup>64</sup> De la Torre et al reported MDMA's rate of activation is decreased when quinidine is added, suggesting MDMA is metabolized in part by CYP2D6.<sup>66</sup> Studies that include CMT used different methodology such as Western blotting with serum containing anti-CYP2D6<sup>67</sup> and use different factors to determine the kinetic parameters for CMT.<sup>29,68</sup> Early studies examining the effects of CMT on CYP2D6 as well as other P450 isozymes describe the binding actions of CMT to CYP2D6 and that this interaction can reduce the metabolism of a benzodiazepine by up to 45%.<sup>69,70</sup> A report by Madeira et al. showed that CMT and DEX could reciprocally interfere with CYP2D6-mediated metabolism.<sup>29</sup>

Literature describing the actions of DEX on CYP2D6 are the most prevalent and use DEX as a probe to determine enzyme activity or phenotyping.<sup>29,41,68</sup> Also DEX has been used to categorize the metabolizer-typing of the patient.<sup>45</sup> Studies on DEX-mediated CYP2D6 inhibition report  $IC_{50}$  values for DEX of 1.89  $\mu$ M to 2.0  $\mu$ M dependent on drug concentration.<sup>48,65</sup> Based on the DEX studies, it is clear that DEX exerts a robust effect at CYP2D6 and could be an important OTC drug when considering potential drug-drug interactions.

#### Inhibition Studies with rodent isozyme: CYP2D2

Rats were treated as described above for 7 days (day 1 to day 7) and then challenged on Day 8 with MA. Following challenge (7 hours), rats were sacrificed and hepatic lobes removed to determine the conversion of AMMC to AMHC. The  $K_m$  ( $\mu$ M) and  $V_{max}$  (pmol of AMHC/mg protein/minute) values are presented in Table 1. Comparison of  $K_m$  values across treatments revealed a significant effect of treatment ( $F_{4,15}=4.067$ ;  $p=0.0199$ ). Although reductions in  $K_m$  ranged from 65-121% compared to naïve values, the actual range was from 3-6.8  $\mu$ M. The saline, CMT, and DEX groups were significantly different from naïve control values ( $p<0.05$ ). The only group that was not statistically different from naïve was the combination of CMT+DEX. It is unclear as to whether these relatively small changes would result in significant changes in metabolism *via* CYP2D2 (or CYP2D6 in humans). Comparing  $V_{max}$  values revealed the more robust changes. Across all treatment groups, it appeared that the CYP2D2 activity had shifted to a higher capacity (280-490%).  $V_{max}$  values were significantly ( $F_{4,15}=4.342$ ;  $p=0.0157$ ) increased compared to naïve values by 2.8- to 4.9-fold. Dunnett's posthoc analysis showed that the only group that was significantly different from naïve was the CMT group ( $p<0.05$ ).

We further confirmed and extended the *in vitro* findings with *in vitro* studies utilizing adult male Sprague-Dawley rats. Rodent CYP2D2 activity following daily drug administration was measured using a modified assay based on the *in vitro* studies. Our *in vivo* results suggest that both  $V_{max}$  and  $K_m$  values were elevated in the treatment groups.  $V_{max}$  values increased 280-490% and  $K_m$  values increased 165-220% in treatment groups compared to  $V_{max}$  and  $K_m$  values from the control (naïve) group. Collectively, these results suggest that following treatment with CMT, DEX or a combination, the CYP2D2 enzyme in the rat shifts towards a low affinity/high capacity CYP2D2 isoform.

**Table 1:** Mean  $K_m$  &  $V_{max}$  Values for each Treatment Groups.

	Naïve	Saline	CMT	DEX	CMT/DEX
$K_m$ ( $\mu$ M) Mean $\pm$ SEM	3.08 $\pm$ 0.46	6.81* $\pm$ 0.73	6.72* $\pm$ 1.34	6.40* $\pm$ 0.47	5.09 $\pm$ 0.55
$V_{max}$ (pmol/mg protein/min) Mean $\pm$ SEM	19.9 $\pm$ 5.1	65.9 $\pm$ 12.0	98.3* $\pm$ 22.1	70.5 $\pm$ 15.1	56.4 $\pm$ 6.1

\* $p<0.05$  compared to corresponding naïve values.

The rightward shift direction of the kinetic curves would lead to a slowing of metabolism over time, and as a result, increasing the concentration of other drugs/compounds which require CYP2D6 for their metabolism. All test compounds reduced the activity of CYP2D6, i.e., weak inhibition; therefore further studies are relevant.

*In vivo* studies showed that pre-treatment with CMT, DEX or CMT+DEX for 7 days followed by an MA challenge on day 8 resulted in CYP2D2 activity that exhibited a lower affinity and higher capacity. This data implies that MA may be an inducer *via* CYP2D2 if elevations in  $V_{\max}$  represent an increase in the quantity of CYP2D2 protein. Conversely,  $V_{\max}$  elevation may be due to changes in the catalytic activity of the existing enzymes. Dostalek et al suggest that MA may be an inducer of DEX metabolism *via* CYP2D2; therefore the co-administration of MA with DEX may result in decreased drug plasma levels thus a decrease in drug effects.<sup>71,72</sup> Conversely, reports have shown that co-administration of DEX and MA reduces the reinforcing properties of MA, *via* inhibition of DA neurons in the brain.<sup>73</sup> This effect could lead to increased MA administration to achieve the same “high,” which will bring plasma levels closer to toxicity. Similar results were reported by Glick et al and these findings also extended DEX and DXO effects to include reductions in both morphine and nicotine self-administration as well as MA.<sup>42</sup> Studies on MDMA metabolism are more recent, but tend to support previous findings with other MA analogs. Although CYP2D6 is the major route of metabolism, other P450 isozymes contribute to the overall metabolism of MDMA.<sup>11,12,74,75</sup> A recent study examined the potential drug-drug interactions between MDMA and caffeine.<sup>76</sup> Although, the authors report that there are no drug-drug interactions which significantly alter MDMA metabolism, the authors acknowledge the importance of the investigation into drug-drug interactions. DEX effects can be extended to the central nervous system where reductions in self-administration and neuroprotection are believed to be through non-NMDA glutamate receptor-mediated functions.<sup>42,77,78</sup> The  $K_m$  value in the control (naïve) group was consistent with the suggested  $K_m$  value reported by the manufacturer (BD Biosciences). This suggests that the results from both assays are relevant to each other and can be compared. Most studies that measure enzyme activity of CYP2D6 or CYP2D2 use DEX as the substrate,<sup>12</sup> but this study uses the reduction of AMMC to AHMC to determine the enzyme activity. A few studies report the kinetic parameters for MA, CMT, and DEX, but most use different methods or *in vitro* instead of *in vivo*.<sup>29,61</sup> Lin et al report  $V_{\max}$  and  $K_m$  values for MA, but the values are for both isomers of MA and both types of reactions (4-hydroxylation and N-demethylation) whereas this study did not differentiate between the two isomers or the two types of reactions.<sup>61</sup> Madeira et al report  $V_{\max}$  and  $K_m$  values for CMT but the study is done *in vitro* instead of *in vivo* and with DEX as the probe.<sup>29</sup>

## CONCLUSION

Poly-drug use is a growing concern due to the potential for

drug-drug interactions and increased risk for severe drug-related toxicity. The current study addressed this question by using the combination of the two scheduled drugs, MA (C-II) and MDMA (C-I), and two common and inexpensive OTC drugs, CMT and DEX. CYP2D6 is an important area of research due to the large role it plays in metabolism, its genetic polymorphisms among humans, and its large number of substrates. There has been some concern regarding the impact of hepatic CYP2D6 interactions since there are other sites that are involved in the metabolism of drugs, such as the intestine, kidney, and brain. The clinically relevant drug, Selegiline, has been used in the treatment of Parkinson’s disease and is metabolized to MA by CYP2D6 and as such may be subject to genetic variations. Benetton et al. report that any P450 phenotyping variations would be insignificant for the metabolism of Selegiline to MA, and this was further extended to include the metabolism of MA and MDMA.<sup>66,79</sup> Regardless, there are variations in CYP2D6 activity and not just in hepatic P450 systems. An understudied area is a role that P450 enzymes have in the brain in the metabolism of centrally-acting drugs.<sup>80,81</sup> The pharmacogenetic impact on the metabolism of MDMA is more pronounced with allele-dependency dictating the rate and extent of metabolism.<sup>82</sup> Although, the mechanisms are not clear, there still exists the change for drug-drug interactions leading to toxicity. Since MA and MDMA are popular and easily obtainable, drug-drug interactions are probable. This study determined that the inhibitor potency of all test compounds and quinidine were relatively the same. It was determined that some maximum test compounds inhibition decreased significantly compared to maximum quinidine and CMT/MDMA inhibition. This suggests that all the test compounds inhibited CYP2D6 activity; one or all of the drugs may not be metabolized as quickly resulting in toxicity of those drugs. The quinidine  $IC_{50}$  value was consistent with reported values. This indicates that the CYP2D6 was performed in accordance with the manufacturer’s specifications. The  $V_{\max}$  value in the CMT treated group increased significantly compared to naïve. The  $K_m$  values in the CMT and saline treated group increased significantly compared to naïve. Both kinetic parameters showed there was an increase after the MA challenge but no effects due to the OTC drugs. This suggests that the low affinity/high capacity CYP2D2 isoform was upregulated meaning that more CYP2D2 was present, suggesting that MA is an inducer *via* CYP2D2. Understanding that MA can induce CYP2D2 and CYP2D6 is important considering the co-administration of other drugs such as DEX, CMT, or MDMA. This information is vital since many other drugs can be used recreationally to achieve a ‘high’ or to combine drugs to potentiate or prolong the high associated with an illicit drug. Increasing our understanding of these drug-drug relationships will aid in our interpretation of forensic findings as well as provide a better foundation for understanding toxicological relationships between various drugs.

## CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest involved with this manuscript.

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