Characterisation of seven medications approved for attention-deficit/hyperactivity disorder using in vitro models of hepatic metabolism

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ABSTRACT

1. The metabolism of most medications approved for the treatment of attention deficit/hyperactivity disorder (ADHD) is not fully understood.
2. In vitro studies using cryopreserved, plated human hepatocytes (cPHHs) and pooled human liver microsomes (HLMs) were performed to more thoroughly characterise the metabolism of several ADHD medications.
3. The use of enzyme-specific chemical inhibitors indicated a role for CYP2D6 in atomoxetine (ATX) metabolism, and roles for CYP3A4/5 in guanfacine (GUA) metabolism.
4. The 4-hydroxy-atomoxetine and N-desmethyl-atomoxetine pathways represented 98.4% and 1.5% of ATX metabolism in cPHHs, respectively. The 3-OH-guanfacine pathway represented at least 2.6% of GUA metabolism in cPHHs, and 71% in HLMs.
5. The major metabolising enzyme for methylphenidate (MPH) and dexamethasphenidate (dMPH) could not be identified using these methods because these compounds were too unstable. Hydrolysis of these medications was spontaneous and did not require the presence of protein to occur.
6. Clonidine (CLD), amphetamine (AMPH), and dextroamphetamine (dAMPH) did not deplete substantially in cPHHs nor HLMs, suggesting that these compounds may not undergo considerable hepatic metabolism. The major circulating metabolites of AMPH and dAMPH (benzoic acid and hippuric acid) were not observed in either system, and therefore could not be characterised. Additionally, inhibition experiments suggested a very minimal role for CYP2D6 in CLD and AMPH metabolism.

Introduction

Attention deficit/hyperactivity disorder (ADHD) is a common and often lifelong disorder that typically occurs with comorbidities such as anxiety, depression, substance use disorders, and bipolar disorders (Drechsler et al. 2020). Pharmacological management of ADHD or these comorbidities can lead to the use of multiple medications at once, which increases the likelihood of drug–drug interactions (DDIs). Common treatments for these psychiatric conditions are often inhibitors or inducers of drug-metabolising enzymes (DMEs), including bupropion and fluoxetine, which inhibit CYP2D6, and many mood stabilisers, which broadly induce cytochrome P450s (CYP450s). In addition to medications prescribed for psychiatric comorbidities, potential DDIs can occur with medications for non-psychiatric conditions as well. A thorough understanding of the pharmacokinetics of a compound can be used to predict pharmacokinetic DDIs and tailor medication regimens for patients with comorbid disorders and, more generally, for those prescribed more than one medication (Wolff et al. 2021). A recent review described the potential for pharmacokinetic-based DDIs in medications approved for ADHD and those in the development pipeline. The authors concluded that the current literature lacked quality and quantity of pharmacokinetic data for certain medications, reducing the ability to inform clinical recommendations (Schoretsanitis et al. 2019). Understanding the DMEs involved in the metabolism of a drug is a critical step towards predicting DDIs and gene–drug interactions (GDIs) in patients carrying genetic variations in DMEs. This can be accomplished through in vitro chemical inhibition studies. In fact, Dückers and Brockmöller (2021) compared the fraction of metabolism predicted from in vitro chemical inhibition...
studies to the impact of DDIs and GDIs in vivo and reported that the results from these types of studies are well correlated. However, this correlation was only observed in substrate depletion studies. The correlation between enzyme fractional clearance in vitro and in vivo was poor when in vitro studies included metabolite formation in the absence of substrate loss and without knowledge of fractional clearance of the pathway. This is problematic for some ADHD medications because the literature has focused on the impact of DMEs in the formation of metabolites, but not on the impact of parent loss. For example, studies have found that cDNA-expressed CYP2D6 4-hydroxylates amphetamine (AMPH) and clonidine (CLD) found that cDNA-expressed CYP2D6 4-hydroxylates amphetamine (AMPH) and clonidine (CLD) in vitro, but these studies do not report substrate clearance (Bach et al. 1999; Claessens et al. 2010).

Therefore, the aim of this study is to build upon the understanding of metabolism for currently approved ADHD medications, including atomoxetine (ATX), guanfacine (GUA), CLD, D/L-AMPH, dextroamphetamine (dAMPH), D/L-methylphenidate (MPH), and dextemethylphenidate (dMPH), using in vitro models of hepatic metabolism. More specifically, the aim is to understand the fraction of metabolism attributed to (1) different metabolic pathways using cryopreserved, primary human hepatocytes (cPHHs) and (2) different enzymes using chemical inhibitors and human liver microsomes (HLMs). cPHHs were used to approximate in vivo hepatic metabolism because they contain all hepatic enzymes and cofactors at physiological concentrations and can remain active throughout a 48-h incubation period, which is important for substrates with low clearances (Jia and Liu 2007). On the other hand, while HLMs contain only a portion of hepatic enzymes, their relative simplicity has allowed the use of specific inhibitors to delineate enzymatic contributions (Lu and Di 2020).

Materials and methods

Test compounds

The following compounds were obtained commercially: (D/L)-AMPH (Cayman Chemical, Ann Arbor, MI), dAMPH (Cayman Chemical, Ann Arbor, MI), 4-hydroxy-amphetamine (4-OH-AMPH; Cayman Chemical, Ann Arbor, MI), norephedrine (Sigma-Aldrich, St. Louis, MO), phenylacetone (Cayman Chemical, Ann Arbor, MI), (D/L)-MPH (Cayman Chemical, Ann Arbor, MI), dMPH (Cerilliant, Round Rock, TX), ritalinic acid (Cayman Chemical, Ann Arbor, MI), ethylphenidate (Cayman Chemical, Ann Arbor, MI), norephedrine (Sigma-Aldrich, St. Louis, MO), phenacetin (Sigma-Aldrich, St. Louis, MO), caffeine (Cayman Chemical, Ann Arbor, MI), dexamfetamine (Cypex, Scotland, UK), diclofenac (TLC Pharmaceutical Standards, Newmarket, Ontario, Canada), benzydamine (Sigma-Aldrich, St. Louis, MO), N-desmethyl-benzydamine (Sigma-Aldrich, St. Louis, MO), and benzydamine-N-oxide (Sigma-Aldrich, St. Louis, MO).

In vitro incubation of pooled, plated, cryopreserved human hepatocytes

The cPHHs used in this study were derived from 30 adult donors (female N = 15; male N = 15; Sekisui XenoTech, Kansas City, KS) and were determined to have normal activity for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5 using various standards metabolised by each enzyme (data not shown). All cPHH experiments were performed by Sekisui XenoTech (Kansas City, KS), and the cPHHs were prepared using vendor protocols (Kazmi, Yerino, Miller, et al. 2014). Briefly, collagen-sandwich hepatocyte cultures with Matrigel overlay underwent a 24–48-h adaptation period using 48-well plates with supplemented Williams’ E medium. Cells were incubated with test articles or control compounds for 0, 4, 8, 24, or 48 h at 37°C while shaking at ~150 rpm under 5% CO₂. The incubation volume was 0.2 mL with ~1.3 × 10⁷ cells/mL. The reaction was stopped with an acetonitrile stop reagent containing an internal standard. The samples were centrifuged at 920 RCF for 10 min at 10°C. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to analyse the supernatant.

The compounds selected for testing were AMPH, dAMPH, MPH, dMPH, ATX, GUA, and CLD. Racemic mixtures of AMPH and MPH were used. All selected compounds were incubated at a concentration of 5 μM, except for racemic mixtures which were incubated together at concentrations of 2.5 μM per enantiomer. These experiments were run in triplicate. To demonstrate the competence of the system, 1 μM S-warfarin and 1 μM diazepam incubated for 0 and 48 h in duplicate were used as positive controls. Negative controls were identical 48-h incubations of the test compound (5 μM) without cPHHs, with sampling at 0 and 48 h in triplicate. This control was used to show that depletion of a test compound was due to the presence of the cPHHs and not due to other incubation conditions.

Pharmacokinetic equations

The elimination rate constant (kₒ) was calculated using the equation of a single exponential decay and first-order...
elimination (Equation 1), where \( C_0 \) is the initial concentration, \( C_t \) is the concentration at a specific timepoint, and \( t \) is that timepoint in minutes.

\[
C_t = C_0 e^{-k_{el}t} \tag{1}
\]

Half-life \((t_{1/2})\) was calculated using the elimination rate constant (Equation 2).

\[
t_{1/2} = \frac{0.693}{k_{el}} \tag{2}
\]

The intrinsic clearance from the metabolic stability in human hepatocytes \((Cl_{int-hepatocytes})\) was calculated using the elimination rate constant \((\text{min}^{-1})\), the incubation volume \((200 \text{ mL})\), and the number of million cells \((10^6 \text{ cells})\) (Equation 3).

\[
Cl_{int-hepatocytes} = \frac{\text{incubation volume}}{\text{number of million cells}} \tag{3}
\]

**In vitro incubation of pooled human liver microsomes**

HLMs from 200 adult donors (female \( N = 100; \) male \( N = 100; \) Sekisui XenoTech XTreme 200 Mixed Gender Human Liver Microsomes) were prepared using vendor protocols. All HLM experiments were performed by Sekisui XenoTech (Kansas City, KS). The pool was evaluated to have normal activity for CYP1A2, CYP2A6, CYP2B6, CYP3A4/5, and CYP3A4/5, flavin-containing monooxygenase (FMOs) and uridine 5'-diphospho-glucuronosyltransferases (UDGTs) using various standards metabolised by each enzyme (data not shown). The incubations included the test compound (5 \( \mu \text{M} \)), a nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system with/without uridine 5'-diphosphoglucuronic acid (UDPGA), and 1\( \text{mg/mL} \) protein content. The test compounds were the same as above. Buffer solutions including potassium phosphate buffer (50 nM, pH 7.4), MgCl\(_2\) (3 nM), and EDTA (1\( \text{mM/L} \), pH 7.4) were incubated at 37°C. The measured incubation times were 0 and 2 h.

**CYP450 phenotyping using chemical inhibition**

For the chemical inhibition experiments, HLMs were incubated at 37°C in triplicate for each solvent. A positive control testing the competence of the system was run in duplicate using 1 \( \mu \text{M} \) midazolam and 0.1 mg/mL protein. Incubation times were 0 and 60 min. Positive controls were also included for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 activity and inhibition (data not shown). The same inhibitor concentration and solvents were used as described above. The probe substrates were 1 \( \mu \text{M} \) phenacetin, 5 \( \mu \text{M} \) efavirenz, 1 \( \mu \text{M} \) diclofenac, 60 \( \mu \text{M} \) S-mephenytoin, 1 \( \mu \text{M} \) dextromethorphan, and 1 \( \mu \text{M} \) midazolam. For most controls except efavirenz and S-mephenytoin, parent depletion was measured via area ratio measurements. For efavirenz and S-mephenytoin, the concentrations of 8-OH-efavirenz and 4-OH-mephenytoin, respectively, were instead measured for changes in metabolite formation. Each positive control was incubated for 60 min, except efavirenz which was incubated for 5 minutes. To demonstrate competence of carboxylesterase 1 (CES1) in this system, 150 \( \mu \text{M} \) of clopidogrel, a CES1 substrate, was incubated for 120 min with 1\( \text{mg/mL} \) protein and 20 min with 0.1 \( \text{mg/mL} \) protein, and the concentration of clopidogrel carboxylic acid was measured. For both conditions, a substantial amount of clopidogrel carboxylic acid was formed (data not shown).

Finally, to attribute metabolism to the presence of active enzymes, a negative control condition for each solvent was included that did not contain any cofactors. These experiments were run in triplicate. A ‘no protein’ control was included as an additional negative control for MPH and dMPH because CES1 does not require cofactors for activity.

**UGT gene family contribution using the absence of UDPGA**

To measure the overall contribution of UGTs to the metabolism of the test compounds, metabolism was also assessed in the presence of NADPH and absence of UDPGA. This was run in triplicate for each solvent.

**FMO gene family contribution using heat-inactivated HLMs**

In addition to measure the impact of CYP450 inhibition and the absence of UDPGA, the contribution of FMO enzymes was measured using heat-inactivated HLMs (Poulsen et al. 1979). Briefly, 10\( \text{mg/mL} \) HLMs in 250 \( \text{nM} \) sucrose were heated to 50°C for 2 min and then chilled in ice. Positive controls were run by measuring the formation of N-desmethyl-benzydamine and benzylamine N-oxide after a 10-min incubation with 500 \( \mu \text{M} \) benzydamine. Approximately 20–30% of CYP450 activity loss in heat-inactivated HLMs is observed by the incubation of N-desmethyl-benzydamine and benzylamine N-oxide after a 10-min incubation with 500 \( \mu \text{M} \) benzydamine. Approximately 20–30% of CYP450 activity loss in heat-inactivated HLMs is observed by the incubation of N-desmethyl-benzylamine formation, and a >90% loss in FMO activity is observed by the reduction in formation of benzylamine N-oxide (Madan et al. 1993). This control was run for every experiment using heat-inactivated HLMs (data not shown).

**Calculations**

The amount of a substance (pmol) was calculated by multiplying the concentration (\( C, \mu \text{M} \)) by the incubation volume (200 \( \mu \text{L} \)) (Equation 4).
The loss and formation rates were calculated using the average amount or area ratio of the parent or metabolite at 0 (A0) and 120 (A120) min, corrected for protein concentration (1 mg/mL) and the length of the experiment (120 min) (Equation 5).

\[
\text{Formation or loss rate} = \frac{A0 - A120}{120} \times \frac{\text{protein concentration}}{	ext{volume}}
\]

These rates were compared between experimental and control conditions to calculate percent inhibition.

**LC-MS/MS**

LC-MS/MS data were processed using Analyst Instrument Control and Data Processing Software (SCIEX, Framingham, MA), MassLynx software with TargetLynx (Waters, Milford, MA), or equivalent. Where reference standards were available, calibration standard curves were used to determine concentration. These curves were created by finding the line of best fit for known concentrations and measured area ratios. The equations were either linear or quadratic and used weighting factors of 1/x or 1/x². In instances where an analyte peak was detected but was below the lower level of quantification, the lower level of quantification was used in calculating percent inhibition. The inhibition was described as greater than or equal to that percent inhibition. In instances where no analyte peak was detected, a value of zero was used in calculating percent remaining or percent inhibition. Reference standards were not available for 4-hydroxy-norephedrine (4-OH-norephedrine), oxo-methylphenidate (oxo-MPH), p-hydroxy-methylphenidate (p-OH-MPH), N-desmethyl-hydroxy-atomoxetine (N-desmethyl-OH-ATX), 2-hydroxy-methyl-atomoxetine (2-OH-methyl-ATX), or 2-[(2,6-Dichlorophenyl)-imino]-imidazolidine-4-one. These metabolites were monitored, and calculations were performed using the analyte/internal standard peak area ratio. Finally, chiral columns (Daicel Chiral Technologies, CHIRALPAK; Phenomenex, Lux) were used to separate the stereoisomers of racemic AMPH and racemic MPH.

Statistical methods

Values given herein are means of triplicate measurements, where available, or duplicates for control conditions.

**Results**

**In vitro metabolism in cryopreserved, human hepatocytes**

For the seven tested compounds, the metabolism over 48 h ranged from no substrate loss to 100% disappearance (0% remaining; Table 1). The ATX half-life was 217 min and the estimated intrinsic clearance was greater than 2.22 μL/min/million cells. ATX was almost completely metabolised by hour 24, with only 0.6% of the substrate remaining (Figure 1(A)). 4-OH-ATX-O-glucuronide was the major metabolite, having formed 984 pmol by hour 24 (Figure 2(A)). Given that 1000 pmol ATX was administered initially and 100% was lost by 48 h, approximately 98.4% of ATX was recovered as 4-hydroxylated glucuronide. Comparatively, the highest amount of N-desmethyl-ATX detected was 14.9 pmol, amounting to approximately 1.5% of the administered amount. 2-OH-methyl-ATX and N-desmethyl-OH-ATX also formed and were detected using their area ratios (data not shown).

There was moderate, non-enzymatic loss of ATX observed without cPHHs where 84.3% remained after 48 h (Supplemental Table 1). However, this loss was negligible compared to the complete disappearance in the presence of cPHHs, and the measured metabolites did not form in this condition. Therefore, it is unlikely that the measured metabolite formation or the majority of substrate loss in cPHHs were due to non-enzymatic degradation.

GUA loss was moderate (30.4%) in the presence of cPHHs and lower (19.4%) when cPHHs were absent (Figure 1(B), Supplemental Table 1). However, 3-OH-GUA did not form in the absence of cPHHs; in the presence of cPHHs, 6.63 pmol formed in 8 h (Figure 2(B)). This shows that metabolite formation could be attributed to the presence of cPHHs. Approximately 252 pmol of GUA was lost over the 48-h incubation and only 2.6% of this substrate loss was recovered as the 3-hydroxylated metabolite. Given the parent loss of <50%, the exact half-life and estimated *in vitro* intrinsic clearance could not be calculated (Table 1).

**Table 1.** Substrate depletion, half-life and intrinsic clearance in cPHHs.

| Substrate | Measured compound | Percent remaining after 48 h | \(T_{1/2}^a\) estimated from exponential decay (min) | Estimated *in vitro* CLint \(^b\) (μL/min/million cells) |
|-----------|-----------------|---------------------------|--------------------------------------|--------------------------------------|
| AMPH      | dAMPH           | 97.9%                     | >2880                                | <0.185                               |
|           | L-AMPH          | 99.1%                     | >2880                                | <0.185                               |
|           | dAMPH           | No loss                   | >2880                                | <0.185                               |
| CLD       | CLD             | No loss                   | >2880                                | <0.185                               |
| MPH       | dMPH            | 0%                        | 217                                  | >2.22                                |
|           | L-MPH           | 0%                        | 72.9                                 | >2.22                                |
|           | dMPH            | 0%                        | 289                                  | 1.85                                 |
| ATX       | ATX             | 0%                        | 217                                  | >2.22                                |
| GUA       | GUA             | 69.6%                     | >2880                                | <0.185                               |

\(^a\)Half-life.

\(^b\)Intrinsic clearance.

AMPH: D/L-amphetamine; ATX: atomoxetine; CLD: clonidine; dAMPH: dextroamphetamine; cPHH: cryopreserved primary human hepatocytes; dMPH: dexamethylphenidate; GUA: guanfacine; L-AMPH: L-amphetamine; L-MPH: L-methylphenidate; MPH: D/L-methylphenidate.
Figure 1. Metabolic stability of seven test compounds over 48 h in cPHHs. Metabolic stability in cPHHs of (A) ATX (5 μM), (B) GUA (5 μM), (C) L-AMPH (2.5 μM) after racemic AMPH administration, (D) dAMPH (2.5 μM) after racemic AMPH administration, (E) dAMPH after dAMPH administration (5 μM), (F) CLD (5 μM), (G) dMPH (2.5 μM) after racemic MPH administration, (H) dMPH (5 μM) after dMPH administration, and (I) L-MPH (2.5 μM) after racemic MPH administration. AMPH: D/L-amphetamine; ATX: atomoxetine; CLD: clonidine; cPHH: cryopreserved primary human hepatocytes; dAMPH: dextroamphetamine; dMPH: dexmethylphenidate; GUA: guanfacine; L-AMPH: L-amphetamine; L-MPH: L-methylphenidate; MPH: D/L-methylphenidate.
CLD, dAMPH, and AMPH had little to no substrate loss (Figure 1(C–F)). However, appreciable metabolism was observed in the positive controls (Supplemental Table 2), and minor metabolite formation was detected for 4-OH-AMPH, norephedrine, 4-OH-norephedrine, 4-OH-CLD, and 2-[(2,6-Dichlorophenyl)-imino]-imidazolidine-4-one. 4-OH-AMPH peaked at 24 h at 8.74 pmol after AMPH administration, and at 48 h at 6.04 pmol after dAMPH administration. Norephedrine peaked at 2.55 pmol 24 h after AMPH administration (Figure 2(C)) and at 2.32 pmol, 24 h after dAMPH administration (Figure 2(D)). 4-OH-norephedrine was also formed. Phenylacetone, a metabolite within the major metabolic pathway of AMPH, was not detected. Finally, 4-OH-CLD peaked at 48 h at 17.3 pmol (Figure 2(G)) and 2-[(2,6-dichlorophenyl)-imino]-imidazolidine-4-one also formed from CLD. None of the metabolites formed in the absence of cPHHs, indicating some enzymatic metabolism of AMPH, dAMPH, and CLD occurred, albeit negligible.

After administration of MPH to cPHHs, dMPH and L-MPH were undetectable after 24 h and exhibited half-lives of 217 and 72.9 min, respectively (Table 1). After dMPH administration, dMPH had a similar half-life of 289 min. Also, greater than 500 pmol of ritalinic acid was detected after MPH and dMPH administration (Figure 2(E,F)). However, not all of the substrate loss could be attributed to the presence of cPHHs, as substantial non-enzymatic degradation of MPH and dMPH was observed (Supplemental Table 1). Coupled with the fact that non-enzymatic degradation led to substantial formation of ritalinic acid (Supplemental Figure 1), the fraction enzymatically-metabolised via these pathways could not be estimated. P-OH-MPH and oxo-MPH also formed only in the presence of cPHHs and were detected using their area ratios (data not shown). Ethylenphenidate was not detected in any condition.

**In vitro enzyme activity in HLMs**

As with cPHHs, substrate loss varied widely across the compounds in HLMs. ATX, GUA, MPH, and dMPH exhibited greater than 10% parent loss, whereas AMPH, dAMPH, and CLD had less than 10% parent loss. A less than 10% parent loss is not appreciable compared to anticipated variability between conditions. Therefore, chemical inhibition data were not calculated for parent compounds that exhibited low turnover.

ATX showed the highest amount of parent loss (86.9%; Table 2), which was inhibited 100% by paroxetine, a potent CYP2D6 inhibitor (Table 3). The formation of 4-OH-ATX, 4-OH-ATX-O-glucuronide, and N-desmethyl-ATX was also inhibited by 86.4%, 93%, and 100%, respectively, by paroxetine (Table 3, Supplemental Table 3). Phencyclidine, a strong CYP2B6 and moderate CYP2D6 inhibitor (Kazmi, Yerino, Ogilvie, et al. 2014; Yang et al. 2016), also decreased the parent loss of ATX and subsequent formation of 4-OH-ATX, 4-OH-ATX-O-glucuronide, and N-desmethyl-ATX by 45%, 29.5%, 30.6%, and 45.4%, respectively (Table 3, Supplemental Table 3). Ketoconazole, a CYP3A4/5 inhibitor, was the only other inhibitor to show a minor impact on ATX metabolism; it inhibited the formation of N-desmethyl-ATX by 24.8%. Additionally, 2-hydroxy-ATX did not have an available reference standard and was therefore detected with four different retention times across experiments. Different inhibition profiles were observed for each retention time, with a moderate effect of ketoconazole and esomeprazole, and a stronger effect of tienilic acid most often observed (Supplemental Table 3).

GUA parent loss averaged 19.9% and was inhibited 100% by ketoconazole (Tables 2 and 3). Production of the major metabolite, 3-OH-GUA, was also inhibited 54.1% by ketoconazole. Notably, 3-OH-GUA formation was decreased 48.8% in heat-inactivated HLMs, which is somewhat higher than expected for a CYP-mediated pathway (<30%), but also considerably lower than expected for a strong FMO substrate (>90%; Table 4). The remaining conditions did not meaningfully impact GUA loss or 3-OH-GUA formation.

CLD loss was 6.4% on average over the 2-h incubation period (Table 2). This corresponds with the finding in cPHHs. Notably, 4-OH-CLD formed in low amounts, and was inhibited 74.6% by paroxetine and 31.4% by phenycyclidine (Table 3). Given that phenycyclidine, along with being a strong CYP2B6 inhibitor, is a moderate inhibitor of CYP2D6, it is likely that only CYP2D6 plays a role in 4-OH-CLD formation and the inhibition by phenycyclidine is due to a lack of enzyme specificity. The remaining conditions did not meaningfully impact 4-OH-CLD formation. Also, the formation of 2-[(2,6-dichlorophenyl)-imino]-imidazolidine-4-one was only observed in cPHHs and not in HLMs. It is possible that the enzyme responsible for its formation is not retained in HLMs or it requires a cofactor other than NADPH and UDPGA.

Similar to the findings in cPHHs, AMPH and dAMPH failed to appreciably metabolise in HLMs (Table 2), although 4-OH-AMPH was formed. After AMPH administration, 4-OH-AMPH formation was inhibited 55.6% by phenycyclidine and 36.8% by paroxetine. After dAMPH administration, 4-OH-AMPH formation was inhibited 50.7% by phenycyclidine. Further, due to the lower limit of quantification, the inhibition by paroxetine was estimated to be ≥63.1% (Table 3). Because both phenycyclidine and paroxetine show a strong effect on 4-OH-AMPH concentration, both CYP2B6 and CYP2D6 may play a role in the formation of 4-OH-AMPH. Neither norephedrine, 4-OH-norephedrine, nor phenylacetone formed in HLMs.

After MPH administration and a 2-h incubation, 10.5% of dMPH and 12.7% L-MPH was lost. Two hours after dMPH administration, 23.5% of dMPH was lost (Table 2). For both MPH and dMPH, an increase in ritalinic acid concentrations was observed after 2 h. Both parent loss and ritalinic acid formation occurred in a cofactor- and protein-independent manner (Supplemental Table 4). Spontaneous hydrolysis was observed in both MPH and dMPH initially and at 2 h. This aligns with the findings in cPHHs. However, after both MPH and dMPH administration, p-OH-MPH and oxo-MPH formation were cofactor- and protein-dependent. While the importance of these metabolites in overall MPH metabolism could not be estimated due to the lack of available reference standards, the impact of chemical inhibition could be...
Figure 2. Formation of metabolites over 48 h in cPHHs. The formation of metabolites over 48 h in cPHHs is shown where reference standards were available. (A) Metabolite formation after ATX administration. (B) Metabolite formation after GUA administration. (C) Metabolite formation after racemic AMPH administration. (D) Metabolite formation after dAMPH administration. (E) Metabolite formation after racemic MPH administration. (F) Metabolite formation after dMPH administration. (G) Metabolite formation after CLD administration. 3-OH-GUA: 3-hydroxy-guanfacine; 4-OH-AMPH: 4-hydroxy-amphetamine; 4-OH-ATX: 4-hydroxy-atomoxetine; 4-OH-ATX-O-gluc: 4-hydroxy-atomoxetine-O-glucuronide; AMPH: D/L-amphetamine; ATX: atomoxetine; CLD: clonidine; cPHH: cryopreserved primary human hepatocytes; dAMPH: dextroamphetamine; dMPH: dexmethylphenidate; MPH: D/L-methylphenidate; GUA: guanfacine; L-AMPH: L-amphetamine; L-MPH: L-methylphenidate; N-desmethyl-ATX: N-desmethyl-atomoxetine.
Table 2. Substrate loss in inhibitor-free HLMs.

| Substrate | Measured compound | Percent substrate loss |
|-----------|-------------------|-----------------------|
| AMPH | 4-OH-AMPH | 1.6 |
| AMPH | L-AMPH | 8.0 |
| dAMPH | 4-OH-AMPH | 4.6 |
| CLD | CLD | 6.4 |
| MPH | dMPH | 10.5 |
| dMPH | L-MPH | 12.7 |
| ATX | ATX | 86.9 |
| GUA | GUA | 19.9 |

*Average percent substrate loss after 2 h in HLMs in the inhibitor-free controls (experiments run in triplicate).

AMP: D/L-amphetamine; ATX: atomoxetine; CLD: clonidine; dAMPH: dextroamphetamine; dMPH: dextroamphetamine; GUA: guanfacine; HLM: human liver microsomes; L-AMPH: L-amphetamine; L-MPH: L-methylphenidate; MPH: D/L-methylphenidate.

Table 3. CYP involvement in metabolism identified by specific inhibitors with HLMs.

| Substrate | Measured compound | CYP<sup>a</sup> |
|-----------|-------------------|----------------|
| AMPH | 4-OH-AMPH | 3A4/5/1A2/2B6/2C9/2C19/2D6 |
| dAMPH | 4-OH-AMPH | 5.7 |
| CLD | 4-OH-CLD | N.I. |
| ATX | ATX | N.I. |
| 4-OH-ATX | N-desmethyl-ATX | 24.8 |
| GUA | GUA | 54.1 |

<sup>a</sup>A change in parent loss was only reported if at least 10% of the substrate was lost in the no solvent control condition (Table 2) and if parent loss and/or metabolite formation required active enzymes, as shown with the negative controls of no cofactor addition and no protein addition (Supplemental Table 3).

AMP: D/L-amphetamine; ATX: atomoxetine; CLD: clonidine; dAMPH: dextroamphetamine; dMPH: dextroamphetamine; GUA: guanfacine; HLM: human liver microsomes; L-AMPH: L-amphetamine; L-MPH: L-methylphenidate; MPH: D/L-methylphenidate.

Table 4. FMO involvement in metabolism identified by heat-inactivated HLMs.

| Substrate | Measured compound | FMO<sup>b</sup> |
|-----------|-------------------|----------------|
| AMPH | 4-OH-AMPH | 2.8 |
| dAMPH | 4-OH-AMPH | N.I. |
| CLD | 4-OH-CLD | 7.9 |
| ATX | ATX | 5.9 |
| 4-OH-ATX | N-desmethyl-ATX | 29.1 |
| GUA | GUA | 48.8 |

<sup>a</sup>A change in parent loss was only reported if at least 10% of the substrate was lost in the no solvent control condition (Table 2) and if parent loss and/or metabolite formation required active enzymes, as shown with the negative controls of no cofactor addition and no protein addition (Supplemental Table 3). For those reasons, amphetamine, dextroamphetamine, clonidine, methylphenidate, dextroamphetamine, dextroamphetamine, and ritalinic acid results are not shown.

<sup>b</sup>Positive findings are only those over 30% due to the mild effect of CYP450 activity following heat inactivation.

AMP: D/L-amphetamine; ATX: atomoxetine; CLD: clonidine; dAMPH: dextroamphetamine; dMPH: dextroamphetamine; FMO: flavin-containing monooxygenase; GUA: guanfacine; HLM: human liver microsomes; N-desmethyl-ATX: N-desmethyl-atomoxetine; N.I.: no inactivation.

Discussion

With the exception of ATX (Sauer et al. 2005) and the recently approved, non-stimulant viloxazine (Case and Reeves 1975; Yu 2020), very little is known about the metabolism of the medications commonly used to treat ADHD. This study aimed to provide a more in-depth understanding of the hepatic metabolism of seven ADHD medications using <i>in vitro</i> models. Most importantly, this study showed significant metabolism of ATX by CYP2D6 and of GUA by CYP3A4/5. The major DME responsible for the breakdown of MPH, AMPH, dAMPH, and CLD could not be identified either due to a lack of parent loss or instability of parent compounds. Nonetheless, valuable information about metabolism formation for these medications is reported here. Further exploration of the metabolic systems for ADHD medications will be important for clinical considerations surrounding DDIs and GDIs.

Atomoxetine

The major metabolising enzyme for ATX was identified as CYP2D6, which is consistent with previous studies. CYP2D6 has been reported to be the major enzyme responsible for the 4-hydroxylation of ATX both <i>in vivo</i> and <i>in vitro</i> (Ring et al. 2002; Sauer et al. 2003). This pathway accounts for about 87% of the dose excreted in the urine in extensive metabolisers and 37% in poor metabolisers (Sauer et al. 2003). Additionally, another pathway of interest is the N-desmethyl-ATX metabolism of ATX at steady state in CYP2D6 poor metabolisers when compared to extensive metabolisers. However, only about 6% of the dose is recovered from this pathway in poor metabolisers and 3% is recovered in extensive metabolisers. This discrepancy was described by the authors as an effect of slow loss of N-desmethyl-ATX. This is similar to findings in this study, as paroxetine fully inhibited the breakdown of N-desmethyl-ATX to N-desmethyl-OMT-X, therefore suggesting that CYP2D6 poor metabolisers were indeed less able to clear N-desmethyl-ATX.

This study found no inhibition of ATX loss and a negligible 9.1% inhibition of N-desmethyl-ATX formation by the measured. Oxo-MPH was either inhibited by ketoconazole or by paroxetine, phenylcycline, and esomeprazole, across the compounds with different retention times (Supplemental Table 3). P-OH-MPH was inhibited by ketoconazole, paroxetine, or phenylcycline, across the compounds with different retention times (Supplemental Table 3). Finally, across multiple retention times, the formation of both p-OH-MPH and oxo-MPH was impacted >30% by heat inactivation after MPH and dMPH administration (Supplemental Table 5).

The absence of UDPGA did not inhibit parent loss or the formation of non-glucuronide metabolites (data not shown). The only compound significantly impacted by the lack of UDPGA was the glucuronide metabolite, 4-OH-ATX-O-glucuronide, which was not detected in the absence of UDPGA.
CYP2C19 inhibitor. The existing literature is mixed, with some but not all studies demonstrating a role for CYP2C19. For example, CYP2C19 was reported to have the highest N-desmethyl-ATX formation rate among recombinant human CYP450 enzymes in two separate studies (Ring et al. 2002; Dinh et al. 2016). Additionally, an in vivo, single dose pharmacokinetic study of CYP2D6*1/*10 subjects reported a significant difference between CYP2C19 poor and extensive metabolisers for ATX clearance, 4-OH-ATX formation, and N-desmethyl-ATX formation (Choi et al. 2014). On the other hand, consistent with the data presented here, Dinh et al. (2016) also examined the effects of CYP2C19 genetic variants in paediatric HLMs and did not find a significant impact of CYP2C19 on N-desmethyl-ATX concentrations. A larger, pharmacokinetic study utilising steady-state dosing is required to clarify the importance of ATX N-demethylation in patients with different CYP2D6 and CYP2C19 phenotypes or in patients taking inhibitors of those enzymes. Overall, this study confirmed that the major in vitro metabolic pathway is 4-hydroxylation of ATX via CYP2D6. Additionally, limited N-demethylation of ATX was observed with no specific enzyme identified.

**Guanfacine**

In this study, 100% of GUA loss was inhibited by ketoconazole, a potent CYP3A4/5 inhibitor. This is consistent with an in vivo DDI study that demonstrated ketoconazole increases GUA exposure (as measured by area under curve) over 3-fold (Intuniv 2019 [package insert]). GUA is a non-stimulant medication approved for the treatment of ADHD for which metabolite identification and profiling in humans has been evaluated in at least two studies (Kiechel 1980; Inoue et al. 2019). Kiechel (1980) reported that approximately 27.6% of an oral dose can be recovered in the urine as unmodified GUA, and the major metabolite in the urine was the glucuronide conjugate of 3-OH-GUA at 34.5%. Comparable results have also been reported more recently (Inoue et al. 2019). Across these studies, 3-hydroxylation of GUA accounts for >30% of drug metabolism.

In this study, 3-OH-GUA in cPHHs peaked at 6.63 pmol and was detected at 149 pmol in HLMs. This represents a small fraction of the total lost amount of GUA in cPHHs (i.e. 2.6% of 252 pmol of GUA lost), but a large portion of the total lost GUA in HLMs (i.e. 71.0% of 210 pmol of GUA lost). This discrepancy is likely due to multiple factors including, but not limited to, a shorter incubation time in HLMs, and the presence or absence of 3’-phosphoadenosine-5’-phosphosulfate, which is a sulfotransferase cofactor. Sulfotransferase activity may be important to metabolic processing of GUA, as the sulphate conjugate of hydroxy-GUA has been previously reported to be the most abundant drug metabolite in the plasma (Inoue et al. 2019).

Taken together, the current results align with previous reports. GUA was primarily metabolised by CYP3A4/5, suggesting a clear route for further examination to parse the role of these enzymes in predicting potential DDIs and GDIs. Additionally, the contrasting results from studies using cPHHs and HLMs demonstrate a benefit of leveraging both techniques.

**Amphetamine, dextroamphetamine**

Very little substrate loss and 4-OH-AMPH formation were observed for AMPH and dAMPH in this study. The formation of 4-OH-AMPH ranged from 3.22 to 7.43 pmol after administration of approximately 1000 pmol of AMPH and dAMPH in cPHHs and HLMs. These data are consistent with a previous study in humans demonstrating that 4-hydroxylation is a minor pathway in the metabolism of AMPH (Dring et al. 1970). This study reported that 1.1–3.9% of recovered AMPH was detected as 4-OH-AMPH. While a role for CYP2D6 in 4-OH-AMPH formation has been reported (Adderall 2021 [package insert]), only moderate inhibition of 4-OH-AMPH formation by the CYP2D6 inhibitor, paroxetine, was observed in this study (36.8% for AMPH and >63.1% for dAMPH). Additionally, phenycyclidine, a strong CYP2B6 and moderate CYP2D6 inhibitor, was found to inhibit 4-OH-AMPH to a similar or greater extent than paroxetine (55.6% for AMPH and 50.7% for dAMPH). Others have shown that formation of 4-OH-AMPH in the presence of expressed CYP2D6 enzymes is also limited (Bach et al. 1999). These results suggest a minor role for CYP2D6 and a potential role for CYP2B6 in 4-hydroxylation of AMPH and dAMPH.

Notably, benzoic acid, formed from the major metabolite, phenylacetone, did not form in either cPHHs or HLMs in this study. This may suggest that the required enzyme is extrahepatic, the breakdown of AMPH is non-enzymatic, or the assay conditions were not appropriate for the responsible enzyme. One frequently reported variable associated with AMPH metabolism is urinary pH, which is a critical factor in the amount of AMPH recovered in the urine either as the parent drug or as a metabolite (Beckett et al. 2011; Davis et al. 1971; Wan et al. 1978). Therefore, drugs or conditions that alter urinary pH may significantly interact with AMPH excretion, which can be assessed via physiologically-based pharmacokinetic models (Huang et al. 2020). It is possible that the assay conditions reported here are incompatible with the formation of phenylacetone or benzoic acid, and there are numerous plausible explanations. More research is needed to discover the mechanism responsible for the majority of AMPH and dAMPH metabolism and to further clarify the role of CYP2D6.

**Clonidine**

This study reports very little CLD loss and sparse 4-OH-CLD formation in cPHHs and in HLMs. In vivo studies have reported similar results, wherein CLD is excreted 40–60% in the urine unchanged (Kapvay 2020 [package insert]). While previous in vitro studies with recombinant enzymes demonstrated that CYP2D6 metabolises CLD to 4-OH-CLD, these studies did not account for the loss of parent drug and thus may not be reflective of in vivo metabolism (Claessens et al. 2010; Li et al. 2019). This study demonstrates that 4-hydroxylation of CLD was inhibited 74.6% by CYP2D6 inhibition. These findings align with both observations above that CLD...
may not undergo major hepatic metabolism and that 4-hydroxylation is only a minor hepatic pathway that is mediated by CYP2D6. To understand if a meaningful CYP2D6 metabolic pathway exists in vivo, a pharmacokinetic and mass-balance study in a healthy population should be conducted using up to date LC-MS/MS technology with specific attention to quantifying parent and metabolite concentrations in plasma, urine, and faeces over time.

**Methylphenidate, dexmethylphenidate**

In this study, MPH and dMPH were too unstable in incubation conditions to analyse and were rapidly degraded to ritalinic acid. One possible explanation for the non-enzymatic degradation is that the pH of the incubation was sufficient to cause spontaneous hydrolysis to ritalinic acid. In this study, the incubation buffer had a pH of 7.4 for both cPHHs and HLMs. A recent study showed that, as the pH of a bacterial growth medium increased, the hydrolysis of MPH also increased (Aresti-Sanz et al. 2021). Given the pH used in this study, it is reasonable to assume that the pH may have been a contributor to MPH loss and ritalinic acid formation.

Interestingly, the difference in the half-lives of dMPH (217 and 289 min⁻¹) and L-MPH (72.9 min⁻¹) observed in cPHHs match in vitro reports that CES1 has a greater catalytic efficiency for L-MPH than dMPH and in vivo reports that L-MPH has a significantly faster clearance than dMPH (Kimko et al. 1999; Sun et al. 2004).

In vivo, the carboxylase CES1 is responsible for the formation of ritalinic acid from L-MPH and D-MPH and genetic variants in this enzyme can cause significant increases in methylphenidate exposure (Stage et al. 2017, 2019). While CES1 was shown to be active in these experiments by the conversion of clopidogrel to clopidogrel carboxylic acid, the role of spontaneous hydrolysis cannot be discounted. However, p-OH-MPH and oxo-MPH required cofactors and the presence of cPHHs and HLMs to form, indicating an enzymatic origin. More specifically, quantification of these metabolites indicated that they may be formed by CYP3A4/5, CYP2D6, and CYP2B6. However, these metabolites and the enzymes that form them may not be clinically meaningful in vivo. An in vivo study of two subjects showed that 80% of drug-related material in the urine was recovered as ritalinic acid and less than 2% was recovered as both p-OH-MPH and oxo-MPH when 80–90% of the total dose was recovered (Faraj et al. 1974).

**Limitations**

This study is limited since it includes only in vitro characterisation. While this is the first study, to our knowledge, to characterise most stimulant and non-stimulant ADHD medications using in vitro models of hepatic metabolism, it does not replace the need for thorough in vivo studies. HLMs are not perfect representations of hepatic metabolism and cofactors must be experimentally administered; therefore, the translational relevance of this model to the clinical setting may be limited. For example, CLD showed little to no metabolism in cPHHs or HLMs. This may be due to a low hepatic clearance in vivo, or the possibility that CLD requires different experimental conditions to be cleared in vitro; the latter cannot be addressed until the hepatic clearance of CLD is measured in an in vivo pharmacokinetic study. As another example, the tested AMPHs showed little to no parent loss in HLMs or cPHHs. However, in vivo, AMPH can be metabolised primarily to benzoic or hippuric acid which were not detected in this in vitro study (Beckett et al. 2011; Dring et al. 1970).

Additionally, reference materials are required to measure the true impact of certain metabolic pathways. For example, the formation of p-OH-MPH and oxo-MPH cannot be placed into the context of total drug content because they could only be measured using the area ratios and retention times of the different molecules.

Also, phencyclidine and esomeprazole were used to strongly inhibit CYP2B6 and CYP2C19, respectively, though these drugs also act as moderate inhibitors of CYP2D6 and CYP3A4/5, respectively (FDA 2021). Therefore, the results from phencyclidine and esomeprazole inhibition should be interpreted alongside results using paroxetine and ketoconazole as inhibitors.

Finally, this study was limited in its description of metabolism by the fact that only adult cPHHs and HLMs were used. As ADHD is both a paediatric and adult disorder, future studies should include metabolism in paediatric-derived systems.

**Future directions**

In conclusion, the data presented here describe the in vitro characterisation of hepatic metabolism of medications prescribed for ADHD. Future in vivo DDI and GDI studies will be required to continue to build upon the understanding of the pharmacokinetics of these medications.

**Disclosure statement**

RL, DL, DH, RD and HJ were employed by Myriad Neuroscience at the time of this study and received salary and stock options. MPD received research support from Alkermes, Allergan, Assurex (now Myriad Neuroscience), Johnson and Johnson, Lundbeck, Otsuka, Pfizer, Sunovion, Supernus, MINH, and PCORI, and has served as a consultant for Alkermes, Allergan, Assurex (Myriad Neuroscience), CMEology, Johnson and Johnson, Medscape, Myriad Genetics, Neurontics, Sage, and Sunovion. JAF has received research support from Healix Pharmaceuticals and Tetra Discovery Partners, Inc. EN has received research funding for a clinical trial with Emalex Pharmaceuticals and has served as a member of the scientific advisory board for Myriad Genetics. ESC, SW, and JL were employed by Myriad Genetics at the time of this study and received salary and stock options.

**Funding**

This study was funded by Myriad Genetics.

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