Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in “K2/Spice”: Identification of novel cannabinoid receptor ligands

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Abbreviations: JWH-018 [1-naphthalenyl-(1-pentyl-I\textit{H}-indol-3-yl]-methanone, AM2201, delta-9-tetrahydrocannabinol (\textit{\Delta}^9-\textit{THC}), synthetic cannabinoids (SCs), Cytochrome P450 (P450), and Human Liver Microsomes (HLM).
Abstract

Abuse of synthetic cannabinoids (SCs), like JWH-018 and AM2201, is increasing at an alarming rate. Although very little is known about the metabolism and toxicology of these popular designer drugs, mass spectrometric analysis of human urine specimens following JWH-018 and AM2201 exposure identified mono-hydroxylated and carboxylated derivatives as major metabolites. The present study extends these initial findings by testing the hypothesis that JWH-018 and its fluorinated counterpart AM2201 are subject to cytochrome P450 (P450)-mediated oxidation, forming potent hydroxylated metabolites that retain significant affinity and activity at the cannabinoid 1 (CB1) receptor. Kinetic analysis using human liver microsomes (HLM) and recombinant human protein identified CYP2C9 and 1A2 as major P450s involved in the oxidation of the JWH-018 and AM2201. In vitro metabolite formation mirrored human urinary metabolic profiles, and each of the primary enzymes exhibited high affinity ($K_m = 0.81$ to 7.3 $\mu M$) and low to high reaction velocities ($V_{max} = 0.0053$ to 2.7 nmole product/min/nmole protein). The contribution of CYP2C19, 2D6, 2E1 and 3A4 in the hepatic metabolic clearance of these synthetic cannabinoids was minimal ($f_m < 0.2$). In vitro studies demonstrated that the primary metabolites produced in humans display high affinity and intrinsic activity at the CB1 receptor, which was attenuated by the CB1 receptor antagonist O-2050. Results from the present study provide critical, missing data related to potential toxicological properties of “K2” parent compounds and their human metabolites, including mechanism(s) of action at cannabinoid receptors.
Introduction

Although Cannabis (also called marijuana) remains one of the most abused drugs in the United States (Banken, 2004; Seely et al., 2012; Seely et al., 2011), synthetic cannabinoids are gaining popularity among recreational drug users as adulterants of herbal products commonly marketed as “K2” or “Spice” (Atwood et al., 2010; Dresen et al., 2010; EMCDDA, 2009; Kudo et al., 1995; Seely et al., 2012; Seely et al., 2011). JWH-018 and AM2201 represent two common derivatives structurally characterized as aminoalkylindoles (Atwood et al., 2010; Compton et al., 1992a; Compton et al., 1992b). These derivatives were first synthesized in the early 1990s to study the pharmacology of the cannabinoid type 1 (CB₁) receptor, the predominant G-protein coupled receptor in the brain, and the cannabinoid type 2 (CB₂) receptor, located on immune cells (Huffman and Padgett, 2005; Mackie, 2008). Receptor binding studies have shown JWH-018 (Atwood et al., 2010; Brents et al., 2011) and other aminoalkylindoles to be high affinity ligands (Thakur et al., 2005; Ward et al., 1990) at CB₁ and CB₂ receptors. Synthetic cannabinoids have potential therapeutic benefits because of their anti-emetic and pain-relieving properties (Seely et al., 2011), however, most synthetic cannabinoids synthesized to date have never been approved for human use because of toxic side effects and undesirable psychoactive properties (Camp, 2011; Wells and Ott, 2011).

While Cannabis use, which contains the natural psychoactive cannabinoid delta-9-tetrahydrocannabinol (Δ⁹-THC), is usually associated with mild symptoms such as appetite stimulation and orthostatic hypotension (Jones, 2002; Randall et al., 2004), “K2” users present to emergency departments with severe adverse effects including hypertension, visual and auditory hallucinations, tachycardia, sinus bradycardia, chest pain, dysrhythmias, seizures, psychosis, intracranial hemorrhage, and even death (Benford and Caplan, 2011; Every-Palmer, 2011;
Lapoint et al., 2011; Muller et al., 2010; Pant et al., 2012; Schneir et al., 2011; Simmons et al., 2011a; Simmons et al., 2011b; Vearrier and Osterhoudt, 2010; Young et al., 2011). Additionally, recent medical reports indicate that chronic “K2” use may lead to the development of tolerance, dependence and withdrawal (Zimmermann et al., 2009), as has been observed with marijuana abuse (Budney and Hughes, 2006).

Little information is known about the metabolism and pharmacology of these emerging drugs of abuse. We and others have identified several hydroxylated metabolites of JWH-018 and AM2201 in humans with a known history of “K2” use, implicating the role of mixed function oxidases, like cytochrome P450s, in the biotransformation of synthetic cannabinoids (Chimalakonda et al., 2011b; Hutter et al., 2012; Moran et al., 2011; Sobolevsky et al., 2010; Wintermeyer et al., 2010; Zhang et al., 2002). Previous reports show that JWH-018 undergoes hepatic metabolism leading to the formation of several naphthalene-, indole-, and alkyl-side chain hydroxylated and carboxylated metabolites (Figure 1), which are subsequently conjugated by UDP-Glucuronosyltransferases (UGTs) and excreted as glucuronic acid conjugates in human urine (Chimalakonda et al., 2011a; Chimalakonda et al., 2011b). Additionally, a recent study by Brents et al. (Brents et al., 2011) demonstrated the ring- and side-chain hydroxylated derivatives of JWH-018 possessed greater in vitro and in vivo pharmacological activity than Δ⁹-THC, indicating that oxidative biotransformation does not terminate the pharmacological activity of JWH-018. Thus, when compared to Δ⁹-THC, the increased severity and frequency of adverse effects of “K2” may be explained, in part, by the generation of biologically active metabolites that may prolong or intensify CB₁ receptor activation. This important observation exemplifies the necessity for characterizing the specific metabolic pathways responsible for the biotransformation of these new, emerging drugs of abuse.
The purpose of this study was to identify specific P450 isoforms responsible for JWH-018 and AM2201 oxidation and to determine if primary human \( \omega \)- and \( \omega \)-1-hydroxylated metabolites of JWH-018 and AM2201 bind to and activate CB\(_1\) receptors. Data reported herein indicate that CYP2C9 and 1A2 are the major human isoforms involved in the oxidation of JWH-018 and AM2201, and that oxidized metabolites of each compound display nanomolar affinity and intrinsic activity at mouse CB\(_1\) receptors.
Materials and Methods

Materials

All chemicals used for this study were of reagent grade or higher. Analytical standards for JWH-018, AM2201, and their hydroxylated and carboxylated metabolites (Figure 1) were kindly provided by Cayman Chemical Co (Ann Arbor, MI). cDNA expressed human recombinant cytochrome P450 enzymes (BD Supersomes™), pooled human liver microsomes (HLM, 50-donor pool), NADPH regenerating system [(20 mM NADP+, 60 mM glucose-6-phosphate (G6P), 60 mM MgCl₂, and 100 U/mL glucose 6-phosphate dehydrogenase (G6PDH)] were purchased from BD Gentest (Woburn, MA). Optima LC-MS grade acetonitrile and ethanol was purchased from Fisher Scientific (Pittsburgh, PA). Reagent grade formic acid (99% pure) was purchased from Acros Organic (Pittsburgh, PA). Deionized water (DI) was purified to 18.2 MΩ-cm resistivity using an ELGA PURELAB Ultra laboratory water purification system (Woodridge, IL). All other chemicals and reagents, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO).

For CB₁ receptor binding and activity assays, all drugs, with the exception of ω-1-OH metabolites of JWH-018 and AM2201 were diluted to a concentration of 10⁻² M with 100% ethanol and stored at -20⁰ C. The ω-1-OH metabolites of JWH-018 and AM2201 were diluted to a concentration of 10⁻² M with 100% dimethyl sulfoxide (DMSO) and stored at -20⁰ C. Δ⁹-THC was supplied by the National Institute of Drug Abuse (NIDA, Bethesda, MD). CP-55,940 and O-2050 were purchased from Tocris Bioscience (Ellisville, MO). GTPγS and GDP were purchased from EMD Chemical (Gibbstown, NJ), and Sigma Aldrich (St. Louis, MO), respectively. Both chemicals were diluted to a stock concentration of 10⁻² M with water and stored at -20⁰ C. [³H]CP-55,940 (174.6 Ci/mmol) used for competition receptor binding was purchased from
PerkinElmer (Waltham, MA) and \[^{35}\text{S}\]GTP\(\gamma\)S (1250 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

**Equipment**

Kinetic analysis was performed using an Applied Biosystems API-4000 Q TRAP tandem mass spectrometer (Carlsbad, CA) interfaced with an Agilent Series 1200 quaternary liquid chromatography system (Santa Clara, CA.). Analyst software (Version 1.5, Life Technologies, Carlsbad, CA) was used to control the overall operation of the HPLC system and the mass spectrometer. For CB\(_1\) receptor binding and intrinsic activity assays, bound radioactivity was determined by Beckman Coulter LS6500 liquid scintillation spectrophotometry (Brea, CA).

**Screening of JWH-018 and AM2201 using human recombinant P450 isoforms and pooled human liver microsomes**

To examine the metabolism of JWH-018 and AM2201, cDNA expressed human recombinant cytochrome P450 enzymes (2 pmoles, CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4, BD Supersomes\textsuperscript{TM}); pooled human liver microsomes (HLM, 50 µg, BD Gentest, Woburn, MA) from a 50 donor pool were assayed for activity towards these compounds. Substrates (JWH-018 and AM2201, 10 µM, final concentration) were added in ethanol and allowed to dry at ambient temperature. Protein was added in the presence of pH 7.4, 0.1 M KPO\(_4\) (final concentration) buffer, NADPH regeneration system [(Solution A: 20 mM NADP\(^+\), 60 mM glucose-6-phosphate (G6P), 60 mM MgCl\(_2\), Solution B: 100 U/mL glucose 6-phosphate dehydrogenase (G6PDH)] to ensure sufficient NADPH to enable cytochrome P450-mediated reactions. Controls omitting the substrate, protein, NADPH were included with each assay. To determine if oxidoreductase (OR) and cytochrome b5 metabolize JWH-018 and AM2201, JWH-018 and AM2201, a 10 µM (final concentration) of each was incubated in presence of OR and cytochrome b5 (50 µg) under
similar assay conditions. Total reaction volume was 50 µL, and all incubations were performed in triplicate. Reactions were initiated by the addition of the NADPH regenerating system, incubated at 37°C for 90 min and terminated by addition of an equal volume of ethanol. Prior to LC-MS/MS analysis, protein and other particulates were precipitated by centrifugation at 20800 g for 5 min.

**Steady state enzyme kinetic assays**

For kinetic analysis, incubation conditions were optimized for time and protein concentration. All reactions were performed within the linear range of metabolite formation and less than 5% of substrate being consumed during the course of the reaction (data not shown). Other than substrate concentrations and incubation times, reaction mixture compositions and analytical methods were identical to those described for screening assays above. Kinetic parameters were determined by incubating recombinant CYP1A2, 2C9, 2C19 (2 pmoles each) and 2D6 (4 pmoles) in the presence of varying concentrations of substrate (JWH-018 and AM2201, 0.05-100 µM) for 10 min at 37°C for CYP1A2, 2C9, 2C19 and 20 min at 37°C for CYP2D6. For reactions using HLM, varying concentrations of substrates (JWH-018 and AM2201, 0.05-100 µM) were incubated with 75 µg of HLM protein for 20 min at 37°C.

**Liquid-chromatography-tandem mass spectrometric (LC-MS/MS) analysis**

Quantification of analytes of interest was performed using positive ion electrospray ionization LC-MS/MS methods previously developed for trace analysis of synthetic cannabinoid metabolites (Chimalakonda et al., 2011b; Moran et al., 2011). For these studies, analytical standards (0.20 to 100 ng/mL) were matrix matched in pooled human urine void of synthetic cannabinoid contamination or in the final *in vitro* reaction mixture (ethanol/water/0.1M pH 7.4 phosphate buffer: 50/30/20%). Analytes of interest were chromatographically separated as
previously described under isocratic conditions using an Agilent Zorbax Eclipse XDB-C18 analytical column (150 x 4.6 mm, 5 μm) heated to 40°C. Mobile phases consisted of 45% A (0.1% formic acid in water) and 55% B (0.1% formic acid in acetonitrile (Chimalakonda et al., 2011b; Moran et al., 2011). The XDB-C18 analytical column was washed and re-equilibrated between each injection by ramping mobile phase B to 95% and then returning the system to initial conditions. The Turbo Ion Spray source voltage was 2500 volts, and source temperature was maintained at 600°C. Nitrogen gas pressures for the GS1 and GS2 source gases, curtain gas, and collision gases were 55.0 cm/S, 55.0 cm/S, 35.0 cm/S, and “high”, respectively.

Confirmation of each metabolite was determined by including specific reaction monitoring-information dependent acquisitions (SRM-IDA) to obtain enhanced product ion spectra (EPI) for each product. Molecule specific parameters for SRM-IDA experiments are listed in Supplemental Table 1. The SRM-IDA transition threshold that triggered EPI experiments ranged from 100 to 4000 counts per second. Resulting EPI mass spectra for QC and unknown specimens were library matched against stored EPI mass spectra previously reported (Chimalakonda et al., 2011b; Moran et al., 2011) or obtained from newly synthesized analytical standards to ensure similar urinary metabolites that may interfere with analysis were fully resolved. Supplemental Figure 1 shows the mass spectra used to confirm the presence of α-1-OH metabolite of AM2201 in each reaction, and identifies the appropriate molecular ion ([MH⁺], m/z 376) and expected product ions characteristic of aminoalkylindoles (m/z 127 and 155). In addition to mass spectra comparisons, all metabolic products were confirmed by matching retention times of analytical standards. LC-MS/MS conditions have previously been shown to resolve each of the metabolites assayed from their corresponding conjugates (Chimalakonda et al., 2011a).
β-Glucuronidase Treatment of Subject Samples

To evaluate human urinary metabolic profiles, 40 µL of human urine were incubated in the presence and absence of β-glucuronidase (Bovine liver, Type B-10, Sigma-Aldrich, St. Louis, MO) at 37 °C, with constant shaking for 60 minutes, by adding 160 µL of 0.1 M pH 5.0 sodium acetate buffer containing β-glucuronidase (1.6 units/µl final concentration). After incubations, 100 µL of the final reaction mixture was spiked with deuterium labeled internal standards available for ω-OH and ω-COOH metabolites (100 ng/mL final concentrations) prior to LC-MS/MS analysis.

Kinetic Data Analysis

Curve-fitting and statistical analyses were conducted utilizing GraphPad Prism® v4.0b (GraphPad Software, Inc.; San Diego, CA). Kinetic constants were obtained by fitting experimental data to kinetic models using the non-linear regression (Curve Fit) function:

The fit of the data for each model was assessed from the standard error, 95% confidence intervals and R² values. Kinetic curves were also analyzed as Eadie-Hofstee plots to support kinetic models. Kinetic constants were reported as the mean ± standard error of triplicate experiments.

CB₁ receptor affinity and intrinsic activity assays

Membrane preparation

Whole brains were harvested from B6SJL mice, snap-frozen in liquid nitrogen, and stored at -80°C. B6SJL mice were chosen as a model system because the sequence similarity between mice and human CB₁ receptor is >85% similar and B6SJL mice have used extensively by our laboratory to study CB₁ receptor-mediated pharmacology of various cannabinoids (Brents...
Crude membrane homogenates were prepared based on established methods (Prather et al., 2000). Brains were thawed on ice, pooled, and suspended in ice-cold homogenization buffer (50 mM HEPES pH 7.4, 3 mM MgCl₂, and 1 mM EGTA). Suspended brains were then subjected to 10 complete strokes employing a 40 mL dounce glass homogenizer, and centrifuged at 40,000 g for 10 min at 4°C. Supernatants were discarded and pellets were resuspended in ice cold homogenization buffer, homogenized, and centrifuged similarly two more times. Following the final centrifugation step, pellets were resuspended in ice-cold 50 mM HEPES, pH 7.4, to a concentration of approximately 2 mg/mL and aliquoted for storage at -80°C. Protein concentration was determined using BCA™ Protein Assay (Thermo Scientific, Rockford, IL).

**CB₁ receptor binding: Competition binding assay**

50 µg of mouse brain membrane homogenates (containing a relatively pure source of CB₁ receptors) were incubated with 0.2 nM of the radiolabeled cannabinoid agonist [³H]CP-55,940 for 90 min at room temperature in an assay buffer containing 5 mM MgCl₂, 50 mM Tris, 0.05% bovine serum albumin (BSA) and increasing concentrations (0.1 nM–10 µM) of JWH-018, AM2201, and their ω-OH (terminal position on the pentyl side chain) and ω-1-OH and ω-COOH metabolites (Figure 1). Assays were performed in triplicate, in a final volume of 1.0 mL, as previously described (Shoemaker et al., 2005). Total binding was defined as the amount of radioactivity observed when 0.2 nM [³H]CP-55,940 was incubated in the absence of any competitor. Nonspecific binding was defined as the amount of [³H]CP-55,940 binding remaining in the presence of 10 µM of the non-radioactive and non-selective CB₁/CB₂ receptor agonist WIN-55,212-2. Specific binding was calculated by subtracting non-specific from total binding. Reactions were terminated by quick filtration through Whatman GF/B glass fiber filters,
followed by five washes with an ice-cold buffer containing 50 mM Tris and 0.05% bovine serum albumin (BSA). Filters were punched out into 7 mL scintillation vials and immersed in 4 mL of ScintiVerse™ BD Cocktail scintillation fluid. After overnight extraction, bound radioactivity was determined by liquid scintillation spectrophotometry. Specific binding is expressed as a percentage (%) of binding occurring in vehicle samples (binding in the absence of any competitor).

**CB₁ receptor functional assay: [³⁵S]GTPγS binding**

[³⁵S]GTPγS binding was performed as previously described with minor modifications (Prather et al., 2000). Each synthetic cannabinoid or metabolite was incubated with 25 µg of mouse brain membrane homogenates, 10 mM GDP, 0.1 nM [³⁵S]GTPγS and assay buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, 20 units/L adenosine deaminase, 0.05% BSA). Assays were performed in triplicate in a final volume of 1 mL for 30 min at 30°C. Total binding was defined as the amount of radioactivity observed when 0.1 nM [³⁵S]GTPγS was incubated in the absence of any cannabinoid. Nonspecific binding was defined as the amount of [³⁵S]GTPγS binding remaining in the presence of 10 µM of non-radioactive GTPγS. Specific binding was calculated by subtracting nonspecific from total binding. Reactions were terminated by quick filtration through Whatman GF/B glass fiber filters, followed by five washes with an ice-cold buffer containing 20 mM HEPES and 0.05% BSA. Filters were punched out into 7 mL scintillation vials and immersed in 4 mL of ScintiVerse™ BD Cocktail scintillation fluid. After overnight extraction, bound radioactivity was determined by liquid scintillation spectrophotometry. The amount of specific [³⁵S]GTPγS binding produced by each drug was normalized by expressing the G-protein activation for each compound as a percent of binding produced by the full CB receptor agonist CP-55,940.
Statistical Analysis

Curve fitting and statistical analyses for competition binding experiments were performed using Graphpad version 4.0 (GraphPad Software Inc., San Diego, CA). The Cheng-Prusoff equation was used to convert the experimental IC$_{50}$ values obtained from competition receptor binding experiments to K$_i$ values, a quantitative measure of receptor affinity (Cheng and Prusoff, 1973). Non-linear regression for one-site competition was used to determine the IC$_{50}$ for competition receptor binding. E$_{\text{max}}$ (a measure of efficacy) was calculated as the percent of specific [$^{35}$S]GTP$\gamma$S binding produced by each drug, relative to that produced by the full CB receptor agonist CP-55,940. Data are expressed as mean ± SEM. A one-way ANOVA, followed by Neuman-Keuls multiple comparison post hoc test, was used to determine statistical significance (P < 0.05) between the groups. All statistical calculations were performed using Graphpad version 4.0 (GraphPad Software Inc., San Diego, CA).
Results

To begin assessing the physiological relevance of HLM reactions, metabolites produced *in vitro* were qualitatively compared to metabolic products excreted in human urine collected from individuals suspected of using JWH-018 or AM2201 (Figure 3). Consistent with previous reports, JWH-018 and AM2201 appear to be metabolized similarly to produce several ring- and alkyl-side chain oxidized metabolites that are excreted in human urine as glucuronic acid conjugates (Chimalakonda et al., 2011b; Moran et al., 2011). It also appears that AM2201 readily undergoes cytochrome P450-mediated oxidative dehalogenation to produce common metabolites with JWH-018, while the ω-1-OH metabolites of each synthetic cannabinoid are distinct. *In vitro* reactions led to the formation of 3 different indole ring-substituted oxidized metabolites (Figure 3), but these metabolites were not detected in the human specimens. The physiological significance of this finding remains to be determined. While it is difficult to extrapolate urinary concentrations to toxicological endpoints, data identify the pentyl alkyl side chain present in both JWH-018 and AM2201 (Figure 1) as the primary site for P450-mediated oxidation in humans.

To further assess metabolic pathways responsible for JWH-018 and AM2201 oxidation, six human recombinant P450 isoforms listed in Figure 2 were screened for their ability to mediate JWH-018 and AM2201 oxidation. All screening assays used a 10 µM substrate concentration to maximize the potential for identifying all oxidized products. Reaction velocities were estimated by determining the concentration of the ω-OH, ω-COOH, ω-1-OH, and the indole ring-oxidized metabolites (Figure 1) using established LC-MS/MS methods, previously validated for trace level quantification (Chimalakonda et al., 2011b; Moran et al., 2011). After normalizing reaction velocities to hepatic abundance (Rowland-Yeo et al., 2003), CYP1A2 and
2C9 exhibited the most activity towards JWH-018 and AM2201 oxidation, while much less contribution from CYP2C19, 2D6, 2E1, and 3A4 was observed (Figure 2). No interfering products were identified in negative control reactions omitting either substrates or cofactor (data not shown). Incubation of JWH-018 with oxidoreductase (OR) and cytochrome b5 led to the formation of JWH-018 5-OH metabolite (~ 12 nM, data not shown). The concentration of unidentified-OH metabolites of JWH-018 and AM2201 reported in Supplemental Tables 1-5 was estimated from standard responses obtained using the JWH-018 ω-OH analytical standard.

Oxidized products produced during reactions with each recombinant protein (Figure 4) were also qualitatively compared to metabolic profiles observed in human specimens (Figure 3). Similar to metabolic profiles observed in human urine specimens, CYP1A2 reactions with JWH-018 produced the ω-OH, ω-COOH, ω-1-OH metabolites along with a single indole ring-oxidized metabolite (Figure 4A). Interestingly, CYP1A2 is the only isoform that produced the ω-COOH derivative in the presence of JWH-018. CYP2C9 produced equivalent levels of the ω-OH and ω-1-OH metabolites (Figure 4B), while CPY2C19 predominantly catalyzed the formation of the ω-1-OH metabolite (Figure 4C). CYP2D6 catalyzes the formation of several indole ring-oxidized derivatives along with the formation of the alkyl side-chain oxidized metabolites (Figure 4D).

CYP1A2, CYP2C9, and CYP2C19 all mediated oxidative dehalogenation reactions in the presence of AM2201 to form the ω-OH and the ω-COOH metabolites (Figure 4E-G), but additionally, the ω-1-OH derivative of AM2201 along with an unidentified-OH metabolite were also formed. In general, incubation of AM2201 with CYP2D6 led to overall lower metabolite formation in comparison to CYP1A2, 2C9, and 2C19, but sufficient activity led to the
identification of ω-OH and the unidentified-OH derivatives as major AM2201 metabolites (Figure 4H).

**Kinetic Analysis**

Screening data indicate a significant role for CYP2C9 and CYP1A2 in the oxidation of JWH-018 and AM2201. Therefore, the kinetic analysis of JWH-018 and AM2201 oxidation in HLM was compared to recombinant CYP2C9 and 1A2 to further assess the overall contribution of these enzymes in synthetic cannabinoid metabolism. The formation of ω-OH and the ω-1-OH metabolites of JWH-018 in HLM exhibited saturable hyperbolic kinetics consistent with Michaelis-Menten kinetic profile (Table 1 and Supplemental Figure 3A), while the kinetic profile for the formation of indole ring-oxidized (metabolites 3-6, Figure 1) and an unidentified-OH derivatives of JWH-018 exhibited Michaelis-Menten or Hill activation kinetics, respectively (Supplemental Table 2 and Supplemental Figure 6A).

Kinetic analysis of JWH-018 oxidation with human recombinant CYP1A2 exhibited classical Michaelis-Menten kinetics for the formation of ω- and ω-1-OH derivatives, with high affinity (low apparent K_m values ranging from 4.7-5.3 µM) and high capacity (V_max : 833-1438 pmole product/min/nmole protein), while the kinetic profile for the formation of ω-COOH metabolite with CYP1A2 exhibited substrate inhibition (SI). The ω- and ω-1-OH metabolite formation with recombinant CYP2C9 exhibited activation kinetics fitting the Hill equation, with high affinity (low apparent K_m values of 0.81-1.3 µM) and high capacity (V_max values of 487-835 pmole product/min/nmole protein) (Table 1 and Supplemental Figure 4).
HLM reactions with AM2201 followed either classical Michaelis-Menten kinetics or a biphasic kinetic profile (Table 2 and Supplemental Figure 3B). Formation of the $\omega$-COOH metabolite common with JWH-018 and the distinct $\omega$-1-OH metabolite of AM2201 followed Michaelis-Menten kinetics, while formation of the $\omega$-OH metabolite exhibited a biphasic kinetic profile, consistent with microsomal enzyme preparations where more than one type of enzyme is responsible for the formation of product.

Kinetic analysis of AM2201 oxidation with human recombinant CYP1A2 exhibited classical Michaelis-Menten kinetics for the formation $\omega$-OH, $\omega$-COOH, $\omega$-1-OH metabolites, with high affinity (low apparent $K_m$ values ranging from 3.6-6.6 $\mu$M) and high capacity ($V_{\text{max}}$ : 90-319 pmole product/min/nmole protein). The $\omega$-OH and $\omega$-COOH metabolite formation with recombinant CYP2C9 exhibited Michaelis-Menten kinetics, with high affinity (low apparent $K_m$ values of 2.4-7.2 $\mu$M) and high capacity ($V_{\text{max}}$ values of 266-309 pmole product/min/nmole protein). However, the $\omega$-1-OH metabolite formation with CYP2C9 followed substrate inhibition kinetics with high affinity (low apparent $K_m$ values of 7.2 $\mu$M) and high capacity ($V_{\text{max}}$ value of 122 pmole product/min/nmole protein) (Table 2 and Supplemental Figure 5).

Although screening data indicate that the contribution of CYP2C19 and CYP2D6 in the hepatic metabolism of JWH-018 and AM2201 is minimal (Figure 2), actual contributions can only be assessed through detailed kinetic evaluations. Largely, the kinetic profiles observed during either JWH-018 or AM2201 oxidation to form the $\omega$-OH, $\omega$-1-OH and $\omega$-COOH metabolites with recombinant CYP2C19 exhibited atypical kinetics. The reason for this is unknown and is a subject of further study. All $K_m$ and $V_{\text{max}}$ values reported in Table 1 are approximated. Concentrations of oxidized metabolites formed via CYP2D6 oxidation was low, but these reactions did exhibit classical Michaelis-Menten kinetics for the formation of $\omega$- and
ω-1-OH derivatives, with high affinity (low apparent $K_m$ values of 2.8-3.2 µM) and low capacity ($V_{max}$ values of 26-43 pmole product/min/nmole protein) when JWH-018 was used as the substrate (Table 1). When AM2201 was used, the same kinetic profiles were observed for each metabolite except for the ω-COOH metabolite. Formation of the ω-COOH metabolite exhibited atypical inhibition kinetics which did not fit the classic competitive/uncompetitive-substrate inhibition models. $K_m$ and $V_{max}$ values presented in Table 2 are approximated.

To determine major biotransformation pathways responsible for JWH-018 and AM2201 oxidation in human liver microsomes, intrinsic clearance ($CL_{int}$, µl/min/mg protein) was estimated for each metabolite. As shown in Table 3, over 90% of JWH-018 is metabolized in human liver microsomes to form the 5-OH, 6-OH, ω-OH, and ω-1-OH metabolites. The intrinsic clearance of AM2201 follows a slightly different pathway, where about 85% is metabolized to only the ω-OH and ω-COOH metabolites. The ω-1-OH and the unidentified-OH metabolites are formed to a much lesser extent.

To better assess the relative contribution of each cytochrome P450 isozyme, intrinsic clearance ($CL_{int}$, µl/min/nmole P450) for the formation of indole-ring, alkyl-side chain, and unidentified-OH metabolites of JWH-018 and AM2201 with recombinant CYP1A2, 2C9, 2C19, and 2D6 were calculated and normalized to hepatic abundance (Proctor et al., 2004; Rowland-Yeo et al., 2003). The metabolic fraction ($f_{met, CYP}$) calculations presented in Table 4 identify CYP2C9 as the primary isozyme responsible for the metabolism of JWH-018 to its ω-, and ω-1-OH derivatives ($f_{met, 2C9} \sim 0.83-0.90$ and $0.48-0.62$, respectively); whereas CYP1A2 appears to be responsible for ω-COOH formation ($f_{met, 1A2} = 1$). CYP2C9 and CYP1A2 also appear to play an important role in oxidizing AM2201 to the corresponding ω-OH, ω-1-OH, and ω-COOH metabolites with $f_{met, 2C9}$ ranging from 0.48-0.76 and $f_{met, 1A2}$ ranging from 0.11-0.49 (Table 5).
Both CYP1A2 and CYP2C9 also appear to catalyze the formation of the unidentified-OH metabolite of AM2201 (fm, 1A2 ~0.42-0.56 and fm, 2C9 ~ 0.29-0.40), but the physiological significance of this metabolite remains to be determined. CYP2C19 and 2D6 contributions toward JWH-018 and AM2201 hepatic oxidation seem to be negligible (Tables 4 and 5). However, CYP2C19 catalyzed some formation of the unidentified-OH metabolite of AM2201 (fm, 2C19 = 0.19) (Supplemental Table 5).

Pharmacological/Toxicological evaluations: CB1 receptor binding and intrinsic activity

To begin understanding the pharmacological and/or toxicological consequences of synthetic cannabinoid metabolism, we employed CB1 receptor binding and intrinsic activity assays to determine if cytochrome P450-mediated biotransformation led to the formation of biologically active intermediates or if oxidation terminated activity at the cannabinoid receptor. We have previously reported the binding affinity for several of the indole ring-oxidized metabolites and that the ω-COOH metabolite of JWH-018 does not bind to the CB1 receptor (Brents et al., 2011). However, the primary metabolites, with the exception of ω-OH derivative, have never been investigated for CB1 receptor binding and intrinsic activity assays. Studies presented here are similar to our previous report (Brents et al., 2011). In addition, these studies showed that JWH-018, AM2201 and their ω- and ω-1-OH metabolites, but not the ω-COOH metabolite, bind to the CB1 receptor with high affinity. JWH-018, AM2201 and its ω- and ω-1-OH metabolites showed high affinity (Ki ~0.4-35 nM), resulting in complete displacement of [3H]CP-55,940 from the CB1 receptor (Figure 5A-B). The relative rank order of binding affinity for all the tested compounds was AM2201 = JWH-018 > JWH-018/AM2201 ω-1-OH = JWH-018 ω-OH. Importantly, the CB1 receptor affinity was higher for JWH-018 and AM2201, and
similar for ω- and ω-1-OH derivatives of each synthetic cannabinoid (Figure 5) when compared to the affinity of Δ⁹-THC (15.3 nM) for CB₁ receptors (Brents et al., 2011).

The ability of JWH-018, AM2201, ω-, ω-1-OH, and ω-COOH metabolites to activate G-proteins after binding to the CB₁ receptors was investigated using the [³⁵S]GTPγS binding assay (Figure 6A). A receptor saturating concentration (10 µM) of CP-55,940, Δ⁹-THC, JWH-018, AM2201, and their ω-, ω-1-OH, and ω-COOH derivatives was used to determine a measure of maximal efficacy produced by activation of CB₁ receptors in mouse brain membranes. CP-55,940, JWH-018, AM2201, JWH-018 ω- and ω-1-OH, and AM2201 ω-1-OH metabolites produced a similar level of G-protein activation (Figure 6A), and in this assay appear to act as full CB₁ receptor agonists, similar to CP-55,940. The intrinsic efficacy (G-protein activation) of JWH-018, AM2201, and their ω- and ω-1-OH metabolites was statistically greater (p <0.05) than that of the partial agonist and principal psychoactive ingredient of marijuana, Δ⁹-THC (Figure 6A). As might be expected based on poor CB₁ receptor affinity, the ω-COOH derivative of JWH-018 did not stimulate G-proteins (Figure 6A). Co-incubation with a receptor saturating concentration (1 µM) of the CB₁ receptor antagonist O-2050 significantly attenuated G-protein activation elicited by all compounds examined, strongly suggesting CB₁ receptor selectivity of these compounds (Figure 6B). Importantly, O-2050 demonstrated no significant effects on basal G-protein activity when administered alone (data not shown). The CB₁ receptor activation elicited by ω- and ω-1-OH metabolites indicates that cytochrome P450-mediated oxidation of JWH-018 and AM2201 leads to the formation of potent and efficacious metabolites that retain significant affinity and activity at CB₁ receptors.
Discussion

In 2005, SC products, marketed as “K2/Spice” emerged as new drugs of abuse and have been gaining popularity worldwide ever since. Today, there are significant public health concerns related to these psychoactive drugs of abuse with 1 in 9 high school students admitting use of “K2” (Hu et al., 2011). Furthermore, there is increasing morbidity and mortality reports related to these potent human cannabinoid receptor agonists, which were originally synthesized to study the CB1 and CB2 receptors and their potential anti-emetic and pain-relieving therapeutic properties. There is an urgent need to elucidate the pharmacology of these substances in humans. Before pharmacokinetic and pharmacodynamic properties can be adequately studied in humans, metabolic pathways leading to metabolism and elimination, and pathways leading to the generation of biologically active metabolites must be delineated. Therefore, the purpose of the present study was to identify specific P450 isoforms responsible for JWH-018 and AM2201 metabolism and to compare the relative affinity and intrinsic activity of the primary human metabolites at CB1 receptors.

After the discovery of Δ9-THC as the primary psychoactive constituent of Cannabis, several studies (Bland et al., 2005; Bornheim et al., 1992; Wall et al., 1983; Watanabe et al., 1995; Watanabe et al., 2007) demonstrated that Δ9-THC is primarily metabolized to a single active metabolite 11-OH- Δ9-THC by CYP2C9 and to various inactive oxidized products by CYP3A4. In contrast, this study identifies CYP2C9 and 1A2, as primary P450 isoforms involved in the oxidation of the psychoactive synthetic cannabinoids JWH-018 and AM2201. In vitro HLM incubation of JWH-018 and AM2201 primarily led to the formation of alkyl-side chain oxidized metabolites that mirrored metabolic patterns observed in human urine specimens collected after suspected JWH-018 or AM2201 use. Indole-ring oxidized metabolites and two
unidentified hydroxylated metabolites were also produced *in vitro* but the physiological significance of these findings remains to be determined. Consistent with data presented in this report, studies have consistently identified the alkyl side chain oxidized metabolites as the primary circulating, and thus potentially bioavailable, derivatives (Kacinko et al., 2011). Human studies have also demonstrated that glucuronic acid conjugation is required for urinary excretion (Chimalakonda et al., 2011b; Moran et al., 2011) of synthetic cannabinoids.

The present study identified CYP2C9 and 1A2 as primary enzymes involved in the hepatic oxidation of JWH-018 and AM2201. CYP2C9 is one of the major human polymorphic enzymes, with the most common allelic variants showing reduced rates of metabolism towards substrates (Ortiz de Montellano, 1995). This enzyme is highly expressed both in the liver and intestine (Paine et al., 2006) and is involved in the metabolism of Δ⁹-THC and about 16% of drugs in current clinical use, such as fluoxetine, escitalopram, citalopram, lithium, valproic acid, aripiprazole, risperidone (Ortiz de Montellano, 1995). Given the high abundance of CYP2C9 in the intestine, it is anticipated that this isoform will be intricately involved in the intestinal metabolism of synthetic cannabinoids taken orally, as well as other routes of exposures. Consideration of oral routes of exposure is important because we have previously shown that ingestion of JWH-018 can lead to rapid and severe toxicity in humans (Lapoint et al., 2011). Toxic drug-drug interactions are also an important concern for public health officials, especially since hepatic intrinsic clearance associated with CYP2C9 metabolism is greater than 50%.

CYP1A2 also contributed significantly to the hepatic metabolism of JWH-018 and AM2201. This isozyme is involved in the biotransformation of 5-10% of drugs in current clinical use and is known to be differentially expressed in humans, suggesting polymorphic control of enzyme activity (Ortiz de Montellano, 1995). Therefore individuals with significantly lower
expression of CYP1A2 would be predicted to have a decreased capacity for synthetic cannabinoid detoxification and may be at increased risks for adverse consequences.

Although the involvement of CYP2C19 and CYP2D6 in the overall hepatic metabolism of JWH-018 and AM2201 is minimal, the physiological importance of these enzymes in other tissues remains to be determined. For example, it is well established that CYP2D6 is involved in the metabolism of many CNS-acting drugs (Ingelman-Sundberg, 2005) and that this P450 is one of the major mixed-function oxidases highly expressed in cerebral cortex, hippocampus, and cerebellum, regions known for high expression of CB1 receptors (Meyer et al., 2007). CYP2D6 is highly polymorphic, with more than 80 variant alleles identified to date, including the nonfunctional CYP2D6*4 null allele, present in 12 to 21% of Caucasians (Ingelman-Sundberg, 2005). Importantly, CYP2D6 is known to metabolize the endocannabinoid anandamide (Snider et al., 2008), which suggests that this enzyme may also interact with other synthetic cannabinoids like JWH-018 and AM2201 in the brain. While speculative, data presented in this report suggests that CYP2D6 may be critical in controlling brain concentrations of parent synthetic cannabinoids and their active metabolites.

Another critical finding presented in this study is that JWH-018, AM2201 and their major hydroxylated metabolites produced in vitro and in humans exposed to these synthetic cannabinoids, bind to and activate CB1 receptors with nanomolar affinity. These data are in stark contrast to binding data for Δ⁹-THC. All Δ⁹-THC metabolites, except 11-OH-Δ⁹-THC, are inactivated by oxidative metabolism, which prevents further CB1 receptor activation (Adams and Martin, 1996). The higher affinity, potency, and efficacy of JWH-018 and AM2201, coupled with its potential metabolism to a number of equally active metabolites, suggests that both acute and chronic effects of “K2” may be intensified when compared to a similar level of exposure to
marijuana. In relation to Δ⁹-THC, the results presented herein suggest that differences in synthetic cannabinoid metabolism may lead to differential pharmacokinetic and pharmacodynamic properties and might offer explanations for the widely observed adverse clinical manifestations of synthetic cannabinoid abuse.

Data from the present study provide critical, missing information related to the metabolism and toxicological properties of “K2” parent compounds and their human metabolites, including mechanism(s) of action at cannabinoid receptor subtypes. The findings of the present study provide the groundwork for studying potential mechanisms of toxicity in humans and establish a basis for developing widely available screening tools for detecting “K2” abuse. Importantly, data demonstrate that the synthetic cannabinoid compounds have a greater toxicity potential in comparison to Δ⁹-THC because of their unique metabolic profiles and drug/metabolite binding characteristics may predispose humans to a constellation of serious adverse effects reported after “K2” use.
Authorship Contributions

Participated in research design: Chimalakonda, C.L. Moran, James, Moran J.H, Radominska-Pandya, Hollenberg, Prather, Brents

Conducted experiments: Chimalakonda

Performed data analysis: Chimalakonda, Bratton, Moran J.H, Radominska-Pandya, Brents

Wrote or contributed to the writing of the manuscript: Chimalakonda, Seely, Bratton, Brents, Moran CL, Endres, James, Hollenberg, Prather, Radominska-Pandya, Moran J.H
References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


Footnotes

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Legends for Figures.

**Figure 1. Structures of JWH-018 and AM2201 and hydroxyl metabolites.** JWH-018 (1) and AM2201 (2) are structurally similar, with AM2201 differing by the presence of terminal alkyl fluorine atom. The ring hydroxylated metabolites shown are: JWH-018 4-OH (3), JWH-018 5-OH (4), JWH-018 6-OH (5), JWH-018 7-OH (6), and alkyl-side chain metabolites JWH-018 ω-OH (7), JWH-018 ω-COOH (8), JWH-018 ω-1-OH (9), and AM2201 ω-1-OH (10).

**Figure 2. Oxidation of JWH-018 (panel A) and AM2201 (panel B) by human recombinant Cytochrome P450s.** Oxidation of JWH-018 (panel A) and AM2201 (panel B) to ω- and ω-1-OH, and ω-COOH metabolites. Activity is normalized to hepatic abundance of specific P450s (Rowland-Yeo et al., 2003) and expressed as pmole product/min/mg protein.

**Figure 3. LC chromatograms of the hydroxyl and carboxyl metabolites of JWH-018 and AM2201 in human liver microsomes and human urine.** Resulting chromatographs produced from incubation of 10 µM JWH-018 (panel A) and AM2201 (panel C) with 75 µg of human liver microsomes (HLM) as explained in Materials and Methods. Resulting chromatographs produced from representative human urine collected after suspected use of JWH-018 (Panel B) or AM2201 (panel D). Chromatographs shown were generated after treating the urine with β-glucuronidase as described in Materials and Methods. The numbers above chromatographic peaks correspond to the hydroxylated and carboxylated metabolites of JWH-018 and AM2201 shown in Figure 1. Concentrations (ng/ml) of respective metabolites in panel B are as follows: JWH-018 ω-OH (8.53 ng/ml), JWH-018 ω-COOH (4.7 ng/ml), and JWH-018 ω-1-OH (9.6 ng/ml). Concentrations (ng/ml) of respective metabolites in panel D are as follows: JWH-018 ω-OH (19.6 ng/ml), JWH-018 ω-COOH (9.2 ng/ml), and JWH-018 ω-1-OH (1.06 ng/ml). Different color tracings are representative of the Specific Reaction Monitoring (SRM).
experiments (Supplemental Table 1) used to detect analytes 1-10 (Figure 1). Specific LC-MS/MS conditions are provided in Materials and Methods.

Figure 4. LC chromatograms of the hydroxyl and carboxyl metabolites of JWH-018 and AM2201 produced from human cDNA expressed recombinant P450s. Resulting chromatographs of hydroxylated and carboxylated metabolites produced from incubation of 10 μM JWH-018 with CYP1A2 (panel A), CYP2C9 (panel B), CYP2C19 (panel C), and CYP2D6 (panel D) and 10 μM of AM2201 with CYP1A2 (panel E), CYP2C9 (panel F), CYP2C19 (panel G), and CYP2D6 (panel H) as mentioned in Materials and Methods. The numbers above chromatographic peaks correspond to the hydroxylated and carboxylated metabolites of JWH-018 and AM2201 shown in Figure 1. Different color tracings are representative of the Specific Reaction Monitoring (SRM) experiments (Supplemental Table 1) used to detect analytes 1-10 (Figure 1). Specific LC-MS/MS conditions are provided in Materials and Methods.

Figure 5. JWH-018, AM2201 and ω- and ω-1-OH metabolites of each bind CB1 receptor with nanomolar (nM) affinity. JWH-018, AM2201 and ω- and ω-1-OH metabolites, but not ω-COOH metabolite, completely displaced the radio-labeled cannabinoid [3H]CP-55,940 from CB1 receptor.

Figure 6. JWH-018, AM2201 and their ω-and ω-1-OH metabolites activate mouse CB1 receptor (panel A). 10 μM JWH-018 and its ω-and ω-1-OH metabolites activated mouse CB1 receptor with greater efficacy than 10 μM Δ⁹-THC. JWH-018, AM2201 and the ω-1-OH metabolites of each displayed full agonist activity comparable to CP-55, 940. The ω-OH metabolite of JWH-018 displayed similar efficacy as the parent drug. No G-protein activity was observed with the ω-COOH metabolite of JWH-018. [³⁵S]GTPγS specific binding is expressed as % of CP-55, 940 specific binding. Values designated with different letters above the error bars
are significantly different (p < 0.05, one-way ANOVA with Neuman-Keuls multiple comparison post hoc Test, n = 3). **G-protein stimulation in mouse brain by JWH-018, AM2201 and their ω- and ω-1-OH metabolites is blocked by CB₁ receptor selective neutral antagonist O-2050 (Panel B).** G-protein activation by 1 µM CP-55, 940 and JWH-018 is blocked to a similar extent by 1 µM O-2050. Significant attenuation of G-protein stimulation by 1 µM AM2201 is blocked by 1 µM O-2050. G-protein stimulation by 1 µM Δ⁹-THC, ω- and ω-1-OH metabolites of JWH-018 and AM2201 is blocked to a similar extent by 1 µM O-2050. [³⁵S]GTPγS specific binding is expressed as % of CP-55, 940. Values designated with different letters above the error bars are significantly different (p < 0.05, one-way ANOVA with Neuman-Keuls multiple comparison post hoc Test, n = 3).
Tables

Table 1. Kinetic parameters for JWH-018 oxidation. “K_m” and “V_max” values are given in µM and pmole/min/nmole protein for recombinant P450 isoforms and pmole/min/mg protein for HLM, respectively.

<table>
<thead>
<tr>
<th>JWH-018 metabolites</th>
<th>JWH-018</th>
<th>JWH-018</th>
<th>JWH-018</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ω-OH</td>
<td>ω-COOH</td>
<td>ω-1-OH</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>V_max</td>
<td>833 ± 25</td>
<td>53 ± 9.4</td>
</tr>
<tr>
<td></td>
<td>K_m</td>
<td>5.3 ± 0.74</td>
<td>2.3 ± 0.80</td>
</tr>
<tr>
<td></td>
<td>Kinetic Fit</td>
<td>M-M</td>
<td>SI</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>V_max</td>
<td>835 ± 19</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>K_m</td>
<td>0.81 ± 0.07</td>
<td>ND</td>
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<td>Kinetic Fit</td>
<td>Hill, n= 2.2</td>
<td>ND</td>
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<tr>
<td>CYP2C19</td>
<td>V_max</td>
<td>535</td>
<td>13161</td>
</tr>
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<td></td>
<td>K_m</td>
<td>82</td>
<td>36648</td>
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<td></td>
<td>Kinetic Fit</td>
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<td>*</td>
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<td>CYP2D6</td>
<td>V_max</td>
<td>26 ± 1.0</td>
<td>ND</td>
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<td></td>
<td>K_m</td>
<td>2.8 ± 0.52</td>
<td>ND</td>
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<td>Kinetic Fit</td>
<td>M-M</td>
<td>ND</td>
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<td>HLM</td>
<td>V_max</td>
<td>3.9 ± 0.25</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>K_m</td>
<td>7.8 ± 1.9</td>
<td>ND</td>
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<tr>
<td></td>
<td>Kinetic Fit</td>
<td>M-M</td>
<td>ND</td>
</tr>
</tbody>
</table>
M-M: Michaelis-Menten; SI: Substrate inhibition; ND: Not detected. *Kinetic model did not fit the classic competitive/uncompetitive-substrate inhibition models; “Vmax” values represent the highest activity measured before inhibition is seen, and “Km” values represent is the lowest concentration of substrate that results in an activity of ½ ‘observed “V_max”.'
Table 2. Kinetic parameters for AM2201 oxidation. “Km” and “Vmax” values are given in µM and pmole/min/nmole protein for recombinant P450 isoforms and pmole/min/mg protein for HLM, respectively.

<table>
<thead>
<tr>
<th>AM2201 metabolites</th>
<th>JWH-018 ω-OH</th>
<th>JWH-018 ω-COOH</th>
<th>AM2201 ω-1-OH</th>
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<tr>
<td>CYP1A2</td>
<td>Vmax</td>
<td>90 ± 5.2</td>
<td>319 ± 12</td>
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<td></td>
<td>Km</td>
<td>4.8 ± 1.2</td>
<td>3.6 ± 0.64</td>
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<td></td>
<td>Kinetic Fit</td>
<td>M-M</td>
<td>M-M</td>
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<tr>
<td>CYP2C9</td>
<td>Vmax</td>
<td>309 ± 12</td>
<td>266 ± 12</td>
</tr>
<tr>
<td></td>
<td>Km</td>
<td>4.9 ± 0.80</td>
<td>2.4 ± 0.52</td>
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<td>Kinetic Fit</td>
<td>M-M</td>
<td>M-M</td>
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<tr>
<td>CYP2C19</td>
<td>Vmax</td>
<td>202 ± 2.9</td>
<td>44 ± 1.5</td>
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<td></td>
<td>Km</td>
<td>6.3 ± 2.1</td>
<td>1.6 ± 0.30</td>
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<td>Kinetic Fit</td>
<td>SI</td>
<td>M-M</td>
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<tr>
<td>CYP2D6</td>
<td>Vmax</td>
<td>24 ± 1.4</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Km</td>
<td>6.5 ± 1.6</td>
<td>24</td>
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<tr>
<td></td>
<td>Kinetic Fit</td>
<td>M-M</td>
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<tr>
<td>HLM</td>
<td>Vmax</td>
<td>2.8 ± 0.3</td>
<td>3.3 ± 0.16</td>
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<td>Km</td>
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<td>Kinetic Fit</td>
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M-M: Michaelis-Menten; SI: Competitive Substrate inhibition; ND: Not detected. *Kinetic model did not fit the classic competitive/uncompetitive-substrate inhibition models. #Kinetic model did not fit classic Hill equation for activation kinetics; “$V_{\text{max}}$” values represent the highest activity measured before inhibition is seen, and “$K_m$” values represent the lowest concentration of substrate that results in an activity of $\frac{1}{2}$ observed “$V_{\text{max}}$”.
Table 3: Intrinsic clearance ($\text{CL}_{\text{int}}$, µl/min/mg protein) and relative percent calculations for each metabolite of JWH-018 and AM2201 produced in pooled human liver microsomes (HLM)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolites</th>
<th>Minor metabolites</th>
<th>Major metabolites</th>
<th>Total</th>
</tr>
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<tr>
<td></td>
<td>$\text{CL}_{\text{int}}$</td>
<td>5-OH</td>
<td>6-OH</td>
<td>7-OH</td>
</tr>
<tr>
<td>JWH-018</td>
<td>(%)</td>
<td>0.520</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>AM2201</td>
<td>(%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

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Table 4. Fractional contribution ($f_{nm\ CYP}$) of recombinant human P450 (rCYP) isoforms during JWH-018 oxidation. Intrinsic clearance ($CL_{int}$) values are given in µL/min/nmole P450 and hepatic abundance values of specific P450s (Proctor et al., 2004; Rowland-Yeo et al., 2003) are given in nmoles/mg protein, respectively.

<table>
<thead>
<tr>
<th>JWH-018 metabolites</th>
<th>$CL_{int}$ in rCYP</th>
<th>Hepatic Abundance</th>
<th>Fractional Contribution of each P450 ($f_{nm\ CYP}$)</th>
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<tbody>
<tr>
<td>CYP1A2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$\omega$-OH</td>
<td>157</td>
<td>0.045-0.052</td>
<td>0.066-0.097</td>
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<td>$\omega$-COOH</td>
<td>23</td>
<td>0.045-0.052</td>
<td>1.0</td>
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<td>$\omega$-1-OH</td>
<td>306</td>
<td>0.27-0.36</td>
<td>0.27-0.36</td>
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<td>CYP2C9</td>
<td></td>
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<td>$\omega$-OH</td>
<td>1035</td>
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<td>$\omega$-COOH</td>
<td>0</td>
<td>0.006-0.014</td>
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<tr>
<td>$\omega$-1-OH</td>
<td>48.7</td>
<td>0.0011-0.0018</td>
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<td>CYP2D6</td>
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<td>$\omega$-OH</td>
<td>9.3</td>
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<td>$\omega$-COOH</td>
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<tr>
<td>$\omega$-1-OH</td>
<td>13.4</td>
<td>0.003-0.005</td>
<td>0.003-0.005</td>
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</tbody>
</table>
Table 5. Fractional contribution ($f_{ms\, CYP}$) of recombinant human P450 (rCYP) isoforms during AM2201 oxidation. Intrinsic clearance ($CL_{int}$) values are given in $\mu$L/min/nmole P450 and hepatic abundance values of specific P450s (Proctor et al., 2004; Rowland-Yeo et al., 2003) are given in nmoles/mg protein, respectively.

<table>
<thead>
<tr>
<th>AM2201 metabolites</th>
<th>$CL_{int}$ in rCYP</th>
<th>Hepatic Abundance</th>
<th>Fractional Contribution of each P450 ($f_{ms, CYP}$)</th>
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<tbody>
<tr>
<td>CYP1A2</td>
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<td></td>
</tr>
<tr>
<td>$\omega$-OH</td>
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<td>$\omega$-COOH</td>
<td>89</td>
<td>0.045-0.052</td>
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<td>$\omega$-1-OH</td>
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<td>$\omega$-OH</td>
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<td>0.67-0.76</td>
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<tr>
<td>$\omega$-COOH</td>
<td>111</td>
<td>0.032-0.073</td>
<td>0.48-0.70</td>
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<td>$\omega$-1-OH</td>
<td>17</td>
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<td>0.36-0.52</td>
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<td>CYP2C19</td>
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<tr>
<td>$\omega$-OH</td>
<td>32</td>
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<td>0.059-0.091</td>
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<tr>
<td>$\omega$-COOH</td>
<td>28</td>
<td>0.006-0.014</td>
<td>0.021-0.030</td>
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<td>$\omega$-1-OH</td>
<td>0.940</td>
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<td>0.003-0.010</td>
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<td>$\omega$-OH</td>
<td>3.7</td>
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<td>0.001-0.005</td>
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<tr>
<td>$\omega$-COOH</td>
<td>0.371</td>
<td>0.009-0.01</td>
<td>0.0005-0.002</td>
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<tr>
<td>$\omega$-1-OH</td>
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Figure 1

Chemical structures of indole-ring hydroxylated metabolites (P450)

1. JWH-018
2. AM2201

Chemical structures of parent drugs

3. JWH-018 4-OH
4. JWH-018 5-OH
5. JWH-018 6-OH
6. JWH-018 7-OH

Chemical structures of alkyl side-chain hydroxylated metabolites (P450)

7. JWH-018 ω-OH
8. JWH-018 ω-COOH
9. JWH-018 ω-1-OH
10. AM2201 ω-1-OH
Figure 4

(A) 20000

CYP1A2

Signal (CPS)

0 5000 10000 15000 20000

0 2 4 6 8 10 12 14

(B) 50000

CYP2C9

Signal (CPS)

0 10000 20000 30000 40000 50000

0 2 4 6 8 10 12 14

(C) 50000

CYP2C19

Signal (CPS)

0 10000 20000 30000 40000 50000

0 2 4 6 8 10 12 14

(D) 3000

CYP2D6

Signal (CPS)

0 500 1000 1500 2000 2500

0 2 4 6 8 10 12 14

(E) 50000

CYP1A2

0 5000 10000 15000 20000 25000 30000 35000 40000 45000 50000

0 2 4 6 8 10 12 14

(F) 1e+5

CYP2C9

0 1e+3 1e+4 1e+5

0 2 4 6 8 10 12 14

(G) 50000

CYP2C19

0 10000 20000 30000 40000 50000

0 2 4 6 8 10 12 14

(H) 20000

CYP2D6

0 5000 10000 15000 20000

0 2 4 6 8 10 12 14

Time (min)
Figure 5

A) JWH-018 ($K_i: 1.3 \pm 0.25 \text{ nM}$)
   JWH-018 $\omega$-OH ($K_i: 35 \pm 6.5 \text{ nM}$)
   JWH-018 $\omega$-COOH ($K_i: > 10000 \text{ nM}$)
   JWH-018 $\omega$-1-OH ($K_i: 15.4 \pm 7.1 \text{ nM}$)

B) AM-2201 ($K_i: 0.395 \pm 0.074 \text{ nM}$)
   AM-2201 $\omega$-1-OH ($K_i: 12.6 \pm 2.3 \text{ nM}$)