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In vitro inhibition of Carboxylesterase 1 by Major Cannabinoids and Selected Metabolites

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Running title: Cannabinoid inhibition of carboxylesterase 1

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Nonstandard abbreviations:

CES1, carboxylesterase 1; THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol; THC-COOH, 11-nor-∆9-tetrahydrocannabinol-carboxylic acid; 11-OH-THC, 11-Hydroxy-∆9-tetrahydrocannabinol; CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; OST, Oseltamivir phosphate; OC, Oseltamivir carboxylate; PBS, Phosphate buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; C_{max}, maximum plasma concentration; DDI, drug-drug interaction.

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Abstract

The escalating use of medical cannabis and significant recreational use of cannabis in recent years has led to higher potential for metabolic interactions between cannabis or one or more of its components and concurrently used medications. Although there have been a significant number of *in vitro* and *in vivo* assessments of the effects of cannabis on cytochrome P450 and UDP-glucuronosyltransferase enzyme systems, there is limited information regarding the effects of cannabis on the major hepatic esterase, carboxylesterase 1 (CES1). In this study, we investigated the *in vitro* inhibitory effects of the individual major cannabinoids and metabolites ∆9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN), 11-nor-∆9-tetrahydrocannabinol-carboxylic acid (THC-COOH), and 11-Hydroxy-∆9-tetrahydrocannabinol (11-OH-THC) on CES1 activity. S9 fractions from human embryonic kidney (HEK 293) cells stably expressing CES1 were utilized in the assessment of cannabinoid inhibitory effects. THC, CBD, and CBN each exhibited substantial inhibitory potency, and were further studied to determine their mechanism of inhibition and kinetic parameters. The inhibition of CES1 by THC, CBD, and CBN was reversible and appears to proceed through a mixed competitive-noncompetitive mechanism. The K_i values for THC, CBD, and CBN inhibition were 0.541, 0.974, and 0.263 µM (0.170, 0.306, and 0.0817 µg/ml), respectively. Inhibition potency was increased when THC, CBD, and CBN were combined. Compared to the potential unbound plasma concentrations attainable clinically, the K_i values suggest a potential for clinically significant inhibition of CES1 by THC and CBD. CBN however, is expected to have limited impact on CES1. Carefully

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designed clinical studies are warranted to establish the clinical significance of these *in vitro* findings.

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Introduction

Cannabis (*Cannabis sativa* L.; marijuana) is the most commonly abused substance globally and in the US (WHO, 2016; SAMHSA, 2017). In 2017, an estimated 26 million Americans aged 12 or older reported current (past month) marijuana use, a significant increase since 2002 (14.6 million) (SAMHSA, 2017). Beyond its widespread recreational use, the use of cannabis or one or more of its components for therapeutic purposes, i.e. “medical cannabis”, has attracted much interest in recent years from both the lay public and medical community. Medical cannabis is most commonly utilized to alleviate the symptoms (e.g. chronic pain, nausea, anorexia, seizure, etc.) associated with cancers, epilepsy, glaucoma, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), and others (Bridgeman and Abazia, 2017). As of November 2018, the medical use of cannabis or its components in the US was legal in 33 US States and the District of Columbia. Furthermore, in June 2018, the US Food and Drug Administration (FDA) approved cannabidiol (CBD) oral solution for the treatment of seizures associated with two rare and severe forms of epilepsy, Lennox-Gastaut syndrome and Dravet syndrome, in patients two years of age and older (FDA News Release, 2018).

With increasing cannabis use, the potential for cannabis or one or more of its physically active constituents (cannabinoids) and their major metabolites interacting with other therapeutic agents also increases. This risk of drug-drug interactions (DDI) could be substantial given that the diseases and conditions most frequently targeted for treatment with medical cannabis are chronic in nature and treated with conventional medications concurrently. A number of studies have been conducted to evaluate the

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effect of cannabis/cannabinoids on major drug metabolizing enzymes (DMEs). *In vitro*, the major cannabinoids ∆9-tetrahydrocannabinol (THC), CBD, and cannabinol (CBN) were found to either inhibit or induce the cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) through various mechanisms and extents. Specifically, CYP3A4/5/7, 2D6, 2C9/19, 2A6, 2B6, 1A1/2, 1B1, and 2J2 were generally inhibited by those cannabinoids (Yamaori et al., 2010; Yamaori et al., 2011a; Yamaori et al., 2011b; Yamaori et al., 2011c; Yamaori et al., 2012; Jiang et al., 2013; Arnold et al., 2018), while CYP2C9 and mRNA of CYP1A1 were induced by THC (Roth et al., 2001; Bland et al., 2005). As for UGT, UGT1A9 was inhibited by both CBD and CBN, and UGT2B7 was inhibited by CBD but induced by CBN (Al Saabi et al., 2013). In clinical assessments, CBD oral solution was reported to significantly elevate blood/plasma/serum concentrations of antiepileptic drugs including clobazam, N-desmethylclobazam (nCLB, the primary active metabolite of clobazam), rufinamide, topiramate, zonisamide, and eslicarbazepine (Geffrey et al., 2015; Gaston et al., 2017; Devinsky et al., 2018). In addition, chronic exposure to smoked cannabis was associated with decreases in the peak plasma concentration of indinavir and an increase in the clearance of theophylline (Jusko et al., 1978; Jusko et al., 1979; Kosel et al., 2002), suggesting induction of the expression of CYP3A4 and CYP1A2.

Besides CYP450 and UGT enzymes, hydrolases are among the most essential enzymes catalyzing the metabolism of numerous frequently prescribed therapeutic agents (Williams et al., 2004; Laizure et al., 2013; Cerny, 2016). Human carboxylesterase 1 (CES1) is the predominant hepatic esterase contributing to 80-95% of total hydrolytic activity in human liver (Imai et al., 2006; Ross and Crow, 2007) and

hydrolyzes therapeutic agents from nearly every drug class formulated as carboxylic acid esters, amides, thioesters, and carbamates, among which specific agents may rely on CES1 for both deactivation/detoxification (e.g. methylphenidate) or metabolic activation (e.g. oseltamivir) (Takai et al., 1997; Satoh and Hosokawa, 2006; Laizure et al., 2013). CES1 also catalyzes the hydrolysis of a large number of endogenous compounds including short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-coenzyme A (CoA) esters (Lian et al., 2018). *In vitro* studies have reported significant inhibition of CES1 by specific therapeutic agents suggesting potential for clinically significant DDIs when used concurrently with other CES1 substrate medications (Fukami et al., 2010; Zhu et al., 2010; Rhoades et al., 2012; Thomsen et al., 2014). Additionally, an expanding number of natural products and traditional medicines have also been implicated as CES1 inhibitors (Wang et al., 2017).

Given the increasing use of medical and recreational cannabis, the potential for DDIs between cannabis and CES1 substrates warranted investigation. In the present study, we assessed the possible influence of three major cannabinoids (THC, CBD, and CBN) and two major metabolites (11-nor-∆9-tetrahydrocannabinol-carboxylic acid (THC-COOH) and 11-Hydroxy-∆9-tetrahydrocannabinol (11-OH-THC) (Fig. 1) on CES1 activity using an *in vitro* CES1 assay system. Also investigated were the mechanisms and the *in vitro* potency of CES1 inhibition.

Materials and Methods

Materials. Oseltamivir phosphate (OST) was purchased from Sequoia Research Products Ltd. (Pangbourne, United Kingdom). Oseltamivir carboxylate (OC) was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Ritalinic acid, Montelukast, THC, CBD, CBN, THC-COOH, and 11-OH-THC were purchased from Cayman Chemical (Ann Arbor, MI). Phosphate buffered saline (PBS) and 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were the products from Thermo Fisher Scientific Inc. (Waltham, MA). All other chemicals and reagents were of the highest analytical grade and were commercially available.

Preparation of cell S9 fractions containing wild-type CES1. Human embryonic kidney cells (Flp-In-293, Invitrogen, Carlsbad, CA) stably expressing wild-type CES1 have been established and described previously (Zhu et al., 2008). Cells from the established cell line were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 100 µg/ml hygromycin B until reaching approximately 90% confluence. Cells were then harvested in PBS and sonicated. Afterwards, the cell lysates were centrifuged at 9000xg for 30 min at 4 °C, from which the supernatant (S9 fraction) was collected and stored in -70°C freezer until use. The total protein concentrations in S9 fractions were determined using a Pierce BCA assay kit (Thermo Fisher Scientific Inc., Waltham, MA).

Metabolite formation. The active OST metabolite OC, a product of OST hydrolysis (Fig. 2), was formed by incubating OST with S9 fractions in 2-ml tubes at a final volume of 100 µl. Various concentrations of substrate and S9 fractions were prepared separately in reaction buffers (PBS with 10 mM HEPES). The reaction was initiated by

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addition of substrate to S9 fractions mixture in a water bath at 37°C. Our preliminary study indicated that the formation of OC from OST was linear over S9 protein concentrations of 0 – 100 µg/ml and over incubation times of 0 – 30 min. Accordingly, a final S9 protein concentration of 20 µg/ml and a 15-min incubation time were chosen as the standard reaction condition for ensuing inhibition studies. After incubation, the reaction was terminated by adding a 4-fold volume of methanol (400 µl) containing 100 nM ritalinic acid as the internal standard. The mixture was centrifuged at 16,100 × g for 5 min, and the supernatant was further diluted with water/methanol before being transferred to HPLC vials for LC-MS/MS analysis.

Screening of selected cannabinoids and their metabolites as CES1 inhibitors. A preliminary screening assay was conducted to identify the selected cannabinoids and metabolites for their potential to inhibit CES1 activity. THC, CBD, CBN, THC-COOH, and 11-OH-THC were screened at a single concentration of 5 µg/ml. We have previously shown the leukotriene receptor antagonist montelukast to significantly inhibit CES1 activity and it was employed as a positive control (10 µM) (Rhoades et al., 2012). The reaction was initiated by mixing S9 fractions (final concentration 20 µg/ml) with OST (final concentration 500 µM) and tested compounds. The final incubation volume was 100 µl with 1% DMSO. Following the incubation at 37°C for 15 min, the reactions were terminated and prepared for the LC-MS/MS analysis as described in the metabolite formation section above.

Evaluation of time-dependent inhibition by selected cannabinoids. To determine whether the inhibition on CES1 by any of the tested cannabinoids (THC, CBD, and CBN) is irreversible (mechanism-based), the inhibitory potency of those cannabinoids

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was evaluated with and without a 30-min pre-incubation of them with S9 fractions before addition of substrate (OST). The samples were divided into experimental and control groups. In the experimental samples, cannabinoid (final concentration 0 – 50 µg/ml [0 – 161 µM], pre-dissolved in 0.5-µl DMSO due to its insolubility in aqueous buffer) was mixed with S9 fractions (final concentration 20 µg/ml) for a 30-min pre-incubation at 37°C. After the pre-incubation, OST (final concentration1 mM) was added into the mixture to initiate the reaction. In the control group, samples were prepared in the same way as the experimental group except that cannabinoid was added together with OST after the pre-incubation. To avoid the potential influence introduced by a varied solvent (DMSO) effect among different samples, the final DMSO concentration (v/v) in all samples were adjusted to 0.5% during the pre-incubation and 1% during the incubation. After incubation for 15 min, the reactions were terminated and the analytical procedures were as described above in the metabolite formation section.

***In vitro* inhibition study with tested cannabinoids.** For determination of the inhibition constant (K_i) and the type of inhibition, kinetic studies were performed with varying concentrations of substrate (0, 100, 500, 1000, 2500, and 5000 µM) and tested cannabinoids (THC, CBD, and CBN concentration, 0 – 10 µg/ml [0 – 32.2 µM]). The pre-mixture contained S9 fractions (final concentration 20 µg/ml) and cannabinoid in reaction buffer, and the reactions were initiated by adding substrate into the pre-mixture. The final incubation volume was 100 µl with 1% DMSO. Following incubation at 37°C for 15 min, the samples were further prepared for the LC-MS/MS assay as described in the metabolite formation section above.

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Assessment of CES1 inhibition by combined cannabinoids. Since multiple cannabinoids are likely to present in the systemic circulation and liver after the more common types of cannabis use and routes of administration (e.g. smoking), we further assessed whether the inhibition on CES1 would be increased by the combination of the major cannabinoid constituents THC, CBD, and CBN. The final concentration of THC, CBD, and CBN utilized in experiments were 0.200 µg/ml (0.636 µM), 0.133 µg/ml (0.424 µM), and 0.133 µg/ml (0.430 µM), respectively. Those concentrations were approximated with consideration to their calculated Ki and potentially achievable plasma concentrations reported in different clinical studies (Ohlsson et al., 1986; Johansson et al., 1987; Huestis et al., 1992). Cannabinoids were prepared in glass vials before mixing with S9 fractions (final concentration 20 µg/ml) to minimize absorption into tube walls. The reaction was initiated by adding OST (500 µM) to pre-mixture of S9 fractions and cannabinoids. The final incubation volume was 100 µl with 1% DMSO. After a 15 min incubation at 37°C, the samples were prepared for LC-MS/MS assay as described in the metabolite formation section above. The degree of inhibition was compared between samples containing individual cannabinoids versus the three combined cannabinoids.

LC-MS/MS analysis. Oseltamivir carboxylate (OC), the active hydrolytic metabolite of OST, was determined using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) coupled to an AB Sciex API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Chromatographic separation was achieved on a C18 reverse phase analytic column (Aqua, 50 × 2.0 mm, 5 µm, Phenomenex Inc., Torrance, CA). A gradient mobile phase was employed, with the aqueous phase containing 0.1%

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formic acid in water, and the organic phase containing methanol. The mobile phase was delivered at a total flow rate of 0.3 ml/min. Mass spectrometric analysis was performed via electrospray ionization in positive mode, and the mass transitions of m/z 285.3 → 138.3 and 220.2 → 84.4 were monitored for OC and ritalinic acid, respectively. The lower limit of quantification (LLOQ) of OC was estimated to be 25 nM. The inter- and intra- day assay precision as well as accuracy were within 10%. A representative chromatogram obtained from incubation of S9 fractions with 100 μM OST was shown in Fig. 3.

Data analysis. In accordance with a previous report (Hoffmann et al., 2009), some spontaneous hydrolysis of OST in aqueous buffer was observed in our study. Therefore, in the kinetic analysis, the metabolite amount formed in control samples without S9 fractions was subtracted from that in samples with the same substrate concentrations.

In the time-dependent inhibition study, metabolite (OC) formation rate was expressed as a percentage ratio (R_v) of the rate in the control sample without inhibitor. The independent variable was inhibitor concentration ([I]). The half maximal inhibition concentration (IC) was estimated by fitting the following equation (Eq.1) into the experimental data using non-linear regression:

$$R_v = 100 \times \left(1 - \frac{I_{max} \cdot [I]^b}{[I]^b + IC^b}\right) \quad (1)$$

Additional iterated parameters were: I_{max}, the maximal degree of inhibition; and b, a shape exponent. The IC_{50} (inhibitor concentration that achieves 50% R_v) was calculated as follows (Eq. 2):

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$$IC_{50} = IC/(2I_{max} - 1)^{1/b} \quad (2)$$

In the *in vitro* inhibition study, the metabolite formation rate (V) was expressed as the dependent variable of the substrate ([S]) and inhibitor ([I]) concentration. A mixed competitive-non-competitive inhibition model derived from Michaelis-Menten kinetics (Eq.3) was utilized to evaluate the type of inhibition and to determine the inhibition constant of tested cannabinoids. Non-linear regression analysis was performed to fit the model into the experimental data:

$$V = \frac{V_{max} \cdot [S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S](1 + \frac{[I]}{\alpha \cdot K_i})} \quad (3)$$

Iterated parameters were: V_{max}, the maximum metabolite formation rate; K_m, the Michaelis-Menten constant; K_i, the inhibition constant for inhibitor; and α, an indicator of the inhibition type (an α approaching positive infinity indicates competitive inhibition, an α equal to 1 indicates non-competitive inhibition, and an α falling in between indicates mixed inhibition). The majority of notations of kinetic parameters and symbols are adapted from the recommendations of Segel (Segel, 1975).

The type of inhibition was also verified by visual inspection of Lineweaver-Burk plots of the experimental data. The data points at lowest OST concentration (100 µM) were not shown in the plots for easier visual assessment of the line cross point and thereby the inhibition type. In addition, the data group with highest CBN concentration (10 µg/ml [32.2 µM]) was excluded because too few metabolites were formed.

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The potential of clinical DDIs was assessed using the predicted ratio of the area under plasma concentration-time curve (AUC) of substrate with the presence of inhibitor over that without. The predicted ratio (R_{AUC}) was calculated as follows (Eq. 4):

$$R_{AUC} = 1 + I_{max,u}/K_i \quad (4)$$

$I_{max,u}$ is the maximal unbound plasma concentration of the inhibitor observed in clinical, and K_i is the estimated inhibition constant *in vitro*.

Software and statistical methods. The parameter estimation was performed in R 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria) using “nls” package with least square approach. The estimation algorithm was Gauss-Newton. The model performance was assessed by diagnostic residual plots, visual check, and certainty of parameter estimates. Data visualization was performed in R 3.4.2 using “ggplot2” package. Lineweaver-Burk plots were made using Microsoft Excel (Redmond, WA).

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Results

Screening of selected cannabinoids and their metabolites. The relative formation velocities of OC in experimental samples were expressed as a percentage of the results observed in the negative control containing only S9 fractions and substrate. Decreased velocities were observed for all tested compounds (THC, CBD, CBN, THC-COOH, 11-OH-THC) indicating their inhibitory effects on CES1 (Fig. 4). At a concentration of 5 µg/ml, inhibition of greater than 90% was observed for THC, CBD, and CBN, while only less than 50% CES1 inhibition was observed for THC-COOH and 11-OH-THC. Considering the degree of systemic exposures to all of the tested compounds in humans after cannabis/cannabinoid administration, only THC, CBD, and CBN were further investigated.

THC, CBD, and CBN reversibly inhibited oseltamivir hydrolysis. The formation rates of OC from OST were decreased by THC, CBD, and CBN in a concentration-dependent manner (Fig. 5). The IC_{50} for each cannabinoid was computed and compared between samples in which the S9 fractions were pre-incubated with cannabinoids for 30 min before addition of substrate (Fig.5A), and those in which cannabinoids were added after the pre-incubation (Fig.5B). A 30-min pre-incubation with any of the tested cannabinoids did not increase their inhibition potency (Table 1), indicating that their inhibition on CES1-mediated OST hydrolysis was not time-dependent.

***In vitro* kinetic study on inhibition of CES1 activity by selected cannabinoids.** An *in vitro* study was conducted at varying concentrations of substrate (OST, 0 – 5000 µM) and tested cannabinoids (0 – 10 µg/ml [0 – 161 µM]). Nonlinear regression

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analysis was performed to characterize the type of inhibition and estimate kinetic parameters (Fig. 6A1, B1, and C1), which were summarized in Table 2. The K_i values for THC, CBD, and CBN inhibition of OST hydrolysis were 0.541, 0.974, and 0.263 μM (0.170, 0.306, and 0.0817 μg/ml), respectively. Based on the K_i values, the rank order of *in vitro* inhibition potency of the tested cannabinoids was CBN > THC > CBD. The values of α and Lineweaver-Burk plots (Fig. 6A2, B2, and C2) indicated that inhibition of CES1 by all three cannabinoids followed a mixed competitive-noncompetitive model, with characteristics more closely resembling a noncompetitive model.

Increased inhibition by combined THC, CBD, and CBN. When THC, CBD, and CBN were incubated individually and in combination with CES1, the combined cannabinoids exhibited higher extent of inhibition than that observed in any individual group (Fig. 7).

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Discussion

Increased medical and recreational use of cannabis/cannabinoids and concurrent use of conventional medications poses a risk of DDI. We evaluated the potential for major cannabinoids and selected metabolites to inhibit CES1. We identified the three major cannabinoids, THC, CBD, and CBN, as potent inhibitors of CES1 *in vitro*. Pre-incubation of S9 fractions with each of the compounds did not result in potentiation of inhibition indicating reversible characteristics. Furthermore, the inhibition mechanisms were revealed via the parameter α using the reversible inhibition model (Eq. 3) as well as visual inspection of Lineweaver-Burk plots. The estimated α values for inhibition of CES1 by THC, CBD, and CBN were 14.0, 5.8, and 12.0, respectively (Table 1), demonstrating that the inhibition type was mixed competitive-noncompetitive with characteristics more closely resembling noncompetitive inhibition for all three cannabinoids. Lineweaver-Burk plots confirmed this result.

The potential for clinically relevant DDI between tested cannabinoids and CES1 substrates was evaluated comparing the *in vitro* inhibition constant (K_i) from our study results to the potentially achievable inhibitor concentrations in humans. A predicted change (R_{AUC}) in the substrate drug AUC by tested cannabinoids was calculated by Eq. 4. According to both FDA and the European Medicines Agency, a R_{AUC} value larger than 1.02 warrants further investigations into the DDI potential using either mechanistic models or formal clinical study (European Medicines Agency, 2012; Food and Drug Administration, 2017). The exposure to the various cannabis constituents as determined by blood sampling of the systemic circulation varies greatly and is influenced by the formulation used, dosing routes (e.g. oral vs sublingual vs smoking, vs vaporizing) and

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dosing schedule (Huestis 2007). Maximal exposure scenarios were assumed in our evaluation where the highest observed plasma concentrations of tested cannabinoids were utilized. Smoking, the most common route of cannabis use, generally renders the highest THC exposures relative to vaporized and orally ingested cannabis/cannabinoids (Grotenhermen, 2003; Huestis, 2007; Lucas et al., 2018). In a clinical study the peak plasma concentrations (C_{max}) of THC in six healthy males averaged 162 ng/ml (0.515 µM) after smoking a cannabis cigarette containing 3.55% THC (Huestis et al., 1992). Similar THC concentration ranges were observed in other studies where THC was administered by smoking cannabis cigarettes (Lindgren et al., 1981; Perez-Reyes et al., 1982; Lee et al., 2015). The contents of CBD and CBN in cannabis preparations were much lower than that of THC (average 4.5% THC, 0.4% CBD, and 0.3% CBN in cannabis during 1993 - 2008) (Mehmedic et al., 2010). After smoking a cigarette containing 19 mg of deuterium-labeled CBD, a mean C_{max} of 110 ng/ml (0.350 µM) was observed in five healthy males (Ohlsson et al., 1986). In a more recently published pharmacokinetic study on CBD oral solution (Epidiolex®), single dose of 1500 – 6000 mg was found to be well tolerated in six healthy subjects in each dose arm, and the mean C_{max} of CBD were measured 292 – 782 ng/ml (0.928 – 2.49 µM) (Taylor et al., 2018). There is a dearth of information available on CBN pharmacokinetics in humans with a few studies reporting low (< 12 ng/ml [0.0387 µM]) or unquantifiable (< LLOQ) blood/plasma concentrations after smoked or vaporized doses (Schwope et al., 2011; Desrosiers et al., 2014; Newmeyer et al., 2016). Johansson *et al.* reported mean CBN C_{max} of 126 ng/ml (0.406 µM) in six healthy males after smoking a cannabis cigarette containing 19 mg of CBN (Johansson et al., 1987). However, since CBN is unlikely to

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be administered or used therapeutically in a relatively pure form, this concentration was not considered clinically achievable for purposes of our assessment. In R_{AUC} calculation, the plasma protein binding of THC and CBD was assumed to be 97.2% and 94%, respectively based on available data (Garrett and Hunt, 1974; Center for Drug Evaluation and Research, 2018). We were unable to locate documentation of CBN plasma protein binding so an empirical range of > 90% was chosen given its similarity in structure and lipophilicity to THC and CBD. Taken altogether, the $I_{max,u}$ (Eq. 4) was calculated as (C_{max}) × (1 – plasma protein binding), and the resulting R_{AUC} for THC, CBD, and CBN were 1.027, 1.057 – 1.153, and < 1.015, respectively. These results suggest that THC and CBD may act as “perpetrator” constituents toward CES1 substrates, but this requires further clinical investigation and confirmation. Regarding CBN, which also exhibited potent *in vitro* inhibitory effects on CES1, it appears less likely to participate in clinically significant DDIs due to its low anticipated exposure in individuals using cannabis for recreational or medical purposes.

Our study also demonstrated increased inhibition of CES1 activity when THC, CBD, and CBN were combined versus individual constituents. Since individuals using cannabis or multi-constituent extracts for medical reasons or on a recreational basis are more likely to be exposed to a number of major cannabinoids rather than single constituents, this approach may provide a better assessment of potential DDI liability.

Exposures to cannabis can vary depending on product content, dosing route and schedule. Even within a single dosing route such as smoking, multiple factors influence cannabinoid exposure including the cannabis potency, number, duration, and spacing of puffs, hold time and inhalation volume (Huestis, 2007). Further, substantial intra- and

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inter-subject variability in cannabinoid exposures has been documented in pharmacokinetic studies. In the present study we estimated the clinical DDI potential by adopting the higher values we found reported in published literature which might be achieved in actual use scenarios.

A further assumption made in our study was that hydrolysis rates of OST represented only CES1 catalytic activity. This was a reasonable assumption since there is minimal native expression of DMEs, including other esterases in the parent embryonic kidney 293 cells from which the CES1 overexpressing cells were made (Bouman et al., 2011). In addition, OST was reported not to interact with either CYPs or UGTs (He et al., 1999), which further validated our assumption.

Our study has several limitations. First, like most *in vitro* assessments, our evaluation and interpretation were based only on reported systemic concentrations of cannabinoids rather than actual tissue concentrations. CES1 is a ubiquitously expressed enzyme with the liver being by far the predominant expression site (Satoh et al., 2002; Fagerberg et al., 2014). Assessing DDIs based on concentrations reaching metabolic sites (i.e. liver) could generally yield more accurate prediction. However, there are almost no data regarding cannabinoid hepatic concentrations in humans beyond limited post-mortem data suggesting certain cannabinoids (i.e. CBD, CBN) attain much higher concentrations in the liver and bile relative to systemic concentrations. (Gronewold and Skopp, 2011; Fabritius et al., 2012). Second, in the inhibition study conducted for Ki determination, all inhibitor concentrations utilized were higher than the estimated Ki. We believe that the relatively large uncertainty around the estimated α was partially due to this limitation, and it could potentially introduce some error in the prediction of inhibition

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at lower inhibitor concentrations. However, the concentrations of THC, CBD, and CBN in the combination assessment were in the range of their Ki and the inhibition results were consistent with our model prediction, which increased the confidence in estimated parameters.

Reversible inhibition was concluded for THC, CBD, and CBN since their IC_{50} did not decrease after a 30-min pre-incubation with S9 fractions. On the contrary, an actual increase of IC_{50} after the pre-incubation was observed. This phenomenon has also been described in previous reports by another group (Yamaori et al., 2010; Yamaori et al., 2011c; Yamaori et al., 2012). The exact cause(s) of this phenomenon are unclear. As a technical note, we have observed that when prepared at lower concentrations (i.e. around their Ki values), THC, CBD, and CBN prepared in plastic tubes exhibited lower inhibition potency as compared to experiments using glass vials. Therefore, absorption of the cannabinoids onto the plastic incubation tube walls was believed to be a likely cause. This phenomenon has previously been noted by other investigators (Garrett and Hunt, 1974; Christophersen, 1986). Therefore, the incubation time was restricted to 15 min to minimize this potential influence.

Finally, an additional issue for consideration of our findings relates to the role of CES1 in the biotransformation of a large number of endogenous compounds. Indeed, CES1 mediated hydrolysis of esters (e.g. cholesteryl esters and triacylglycerols) is increasingly viewed as an important role in lipid metabolism, cholesterol homeostasis, and possibly fatty liver disease (Lian et al., 2018). Though speculative, a sustained inhibition of CES1 activity as a result of chronic exposure to cannabinoids could have implications for these biological processes as well.

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In summary, our study identified THC, CBD, and CBN as potent inhibitors of CES1 *in vitro*, with a reversible inhibition mechanism and mixed competitive-noncompetitive characteristics. The calculated inhibition constant (K_i) values for THC, CBD, and CBN were 0.541, 0.974, and 0.263 μM, respectively. An increase in the inhibition potency was observed when THC, CBD, and CBN were combined for assessment. When compared to the respective cannabinoid C_{max} achievable physiologically, the K_i values suggest that DDI may occur following the concomitant administration of CES1 substrate medications and THC and CBD, but not by CBN.

The determination of whether the observed *in vitro* inhibitory effects of THC and CBD translates into significant, or even detectable clinical DDIs requires further investigation through formal clinical study. Such formal clinical studies would require the utilization of a well-characterized cannabis product, a standardized dosing regimen, and incorporate an appropriate CES1 substrate as a probe therapeutic agent.

Authorship Contributions

Participated in research design: Qian, Wang, and Markowitz.

Conducted experiments: Qian

Performed data analysis: Qian, Wang, and Markowitz

Wrote and contributed to the writing of the manuscript: Qian and Markowitz

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Figure legends

Figure 1. Cannabinoids and selected metabolites chosen for assessment. THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol; THC-COOH, 11-nor-∆9-tetrahydrocannabinol-carboxylic acid; 11-OH-THC, 11-Hydroxy-∆9-tetrahydrocannabinol.

Figure 2. CES1-mediated activation of oseltamivir phosphate to oseltamivir carboxylate.

Figure 3. Chromatogram of oseltamivir carboxylate (peak in blue) from incubation mixtures containing 100 µM substrate (oseltamivir) and 20 µg/ml S9 fractions. Oseltamivir carboxylate was the active metabolite of oseltamivir specifically catalyzed by CES1. Ritalinic acid (peak in red) was utilized as the internal standard.

Figure 4. Screening of selected cannabinoids and their metabolites as potential modifiers of CES1 activity. The substrate (oseltamivir) concentration was fixed at 500 µM. The concentration of all tested compounds was 5 µg/ml. Montelukast (10 µM) served as a positive control. The CES1 activity was expressed as a percentage ratio relative to the control containing no tested compounds. Individual bars represent mean (±SD) of triplicate samples. The shading of the bars indicates the extent of CES1 inhibition. THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol; THC-

COOH, 11-nor-∆9-tetrahydrocannabinol-carboxylic acid; 11-OH-THC, 11-Hydroxy-∆9-tetrahydrocannabinol.

Figure 5. Inhibition curves for tested cannabinoids in time-dependent inhibition study. The tested cannabinoids were added into S9 fractions before (panel A) and after (panel B) a 30-min pre-incubation. The substrate (oseltamivir) concentration was fixed at 1000 μM. The reaction velocity was expressed as a percentage ratio relative to the control containing no inhibitor. Individual points represent mean (±SD) of triplicate samples. THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol.

Figure 6. Kinetic analysis (panel A1, B1, and C1) and Lineweaver-Burk plots (panel A2, B2, and C2) for *in vitro* inhibition of CES1 by tested cannabinoids. CES1 S9 fractions were incubated with oseltamivir in the absence and presence of THC (A), CBD (B), and CBN (C). Individual points represent mean (±SD) of triplicate samples. THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol.

Figure 7. Inhibition effects of combined cannabinoids on CES1 activity. The substrate (oseltamivir) concentration was fixed at 500 μM. The final concentrations of cannabinoids in the combination sample were 0.363 μM THC, 0.424 μM CBD, and 0.430 μM CBN. The CES1 activity was expressed as a percentage ratio relative to the control containing no inhibitors. Individual bars represent mean (±SD) of triplicate

samples. The shading of the bars indicates the extent of CES1 inhibition. THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol.

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Table 1. Effect of preincubation with tested cannabinoids on CES1 activity

Cannabinoids	IC_{50} (μM)		B/A
	Pre-incubation time		
	0 min (A)	30 min (B)	
THC	3.91	11.2	2.85
CBD	7.73	12.1	1.57
CBN	4.03	8.51	2.11

THC, Δ9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol.

All IC_{50} estimates were obtained from nonlinear regression analysis using model described in Eq. 1.

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Table 2. Parameter estimates of *in vitro* inhibition study with tested cannabinoids

Cannabinoids	K_m (μM)	K_i (μM)	α	V_{max} (nmol/min/mg protein)
THC	993 ± 149	0.541 ± 0.081	14.0 ± 9.3	159 ± 10
CBD	1191 ± 159	0.974 ± 0.169	5.8 ± 3.4	181 ± 9
CBN	1399 ± 108	0.263 ± 0.034	12.0 ± 9.8	193 ± 8

THC, ∆9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol. K_m, the Michaelis-Menten constant; K_i, the inhibition constant; α, an indicator of inhibition type; V_{max}, the maximum metabolite formation velocity.

All values were presented as the parameter estimates ± SE (uncertainty) from nonlinear regression analysis using model described in Eq. 3.

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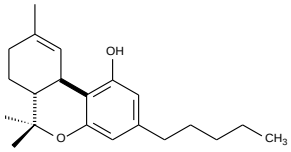
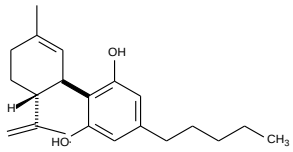
Figure 1



THC



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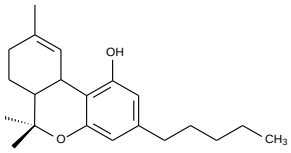

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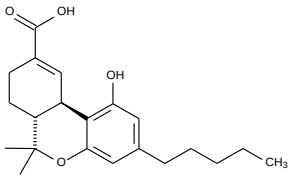
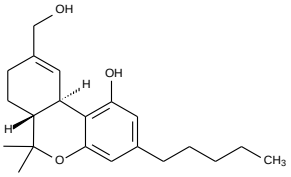


THC-COOH



11-OH-THC

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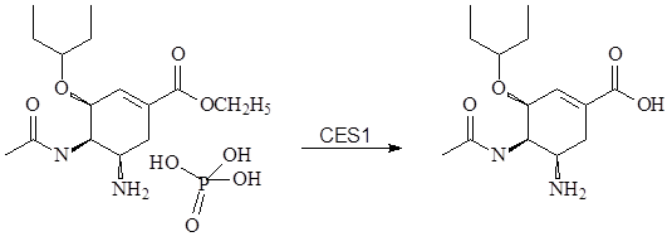
Figure 2



Oseltamivir phosphate

Oseltamivir carboxylate

Figure 3

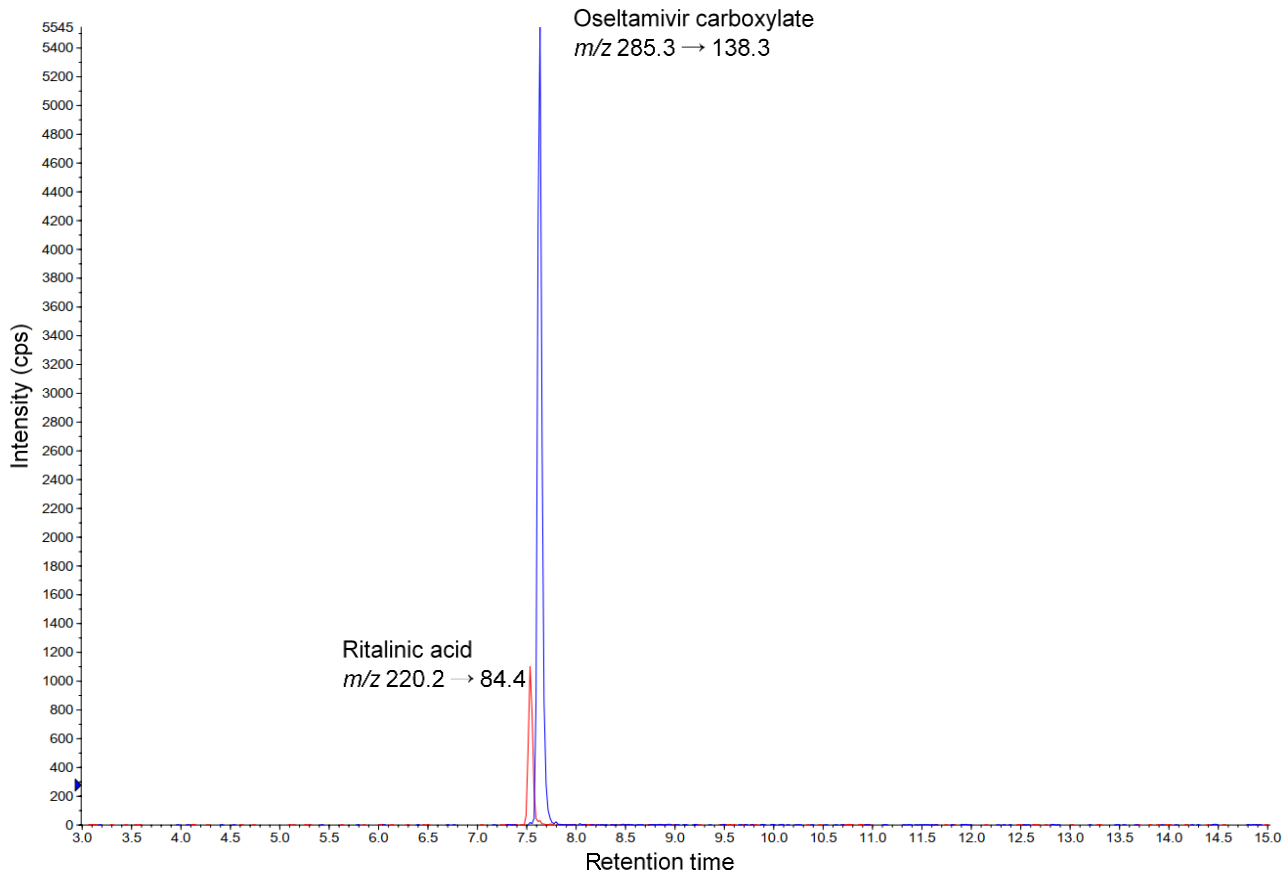


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Figure 4

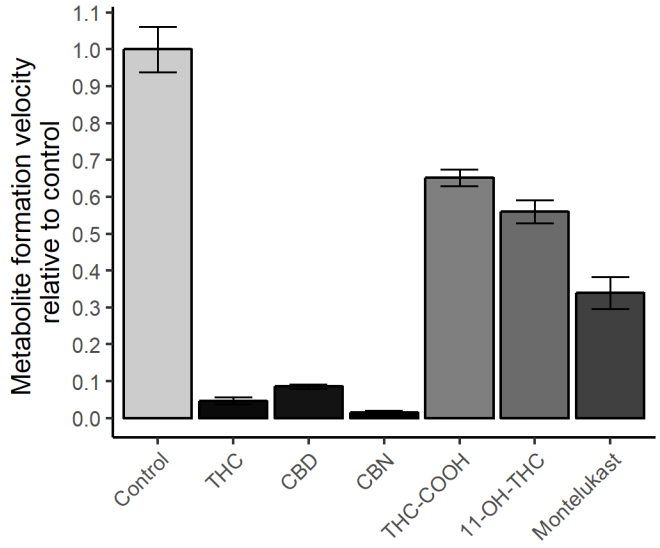

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Figure 5

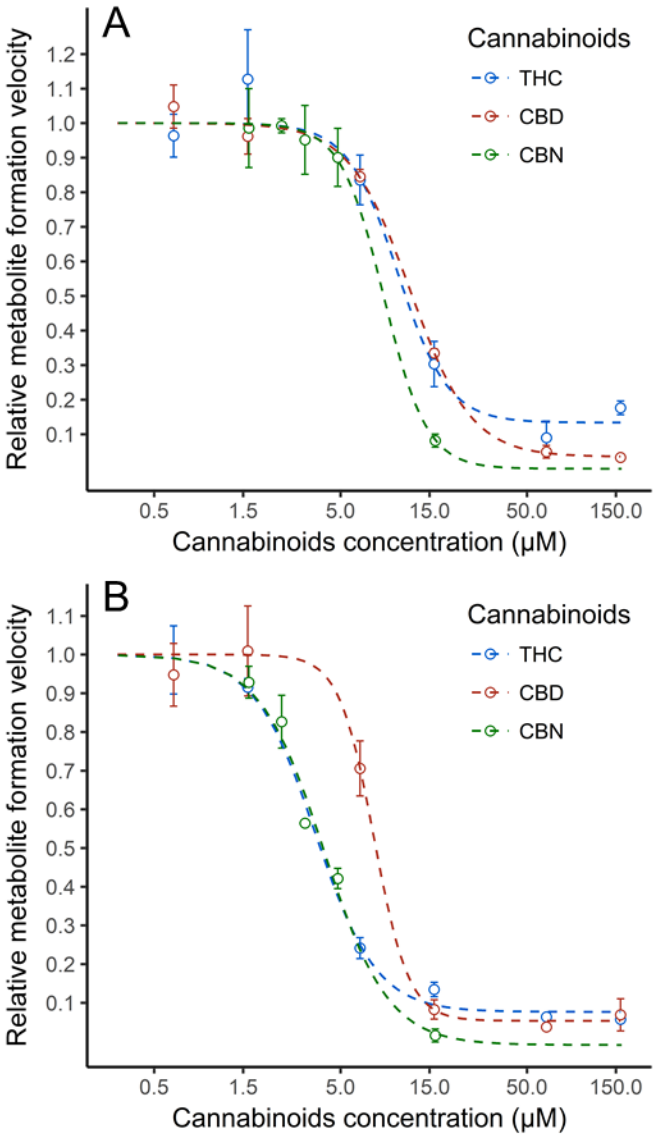

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Figure 6

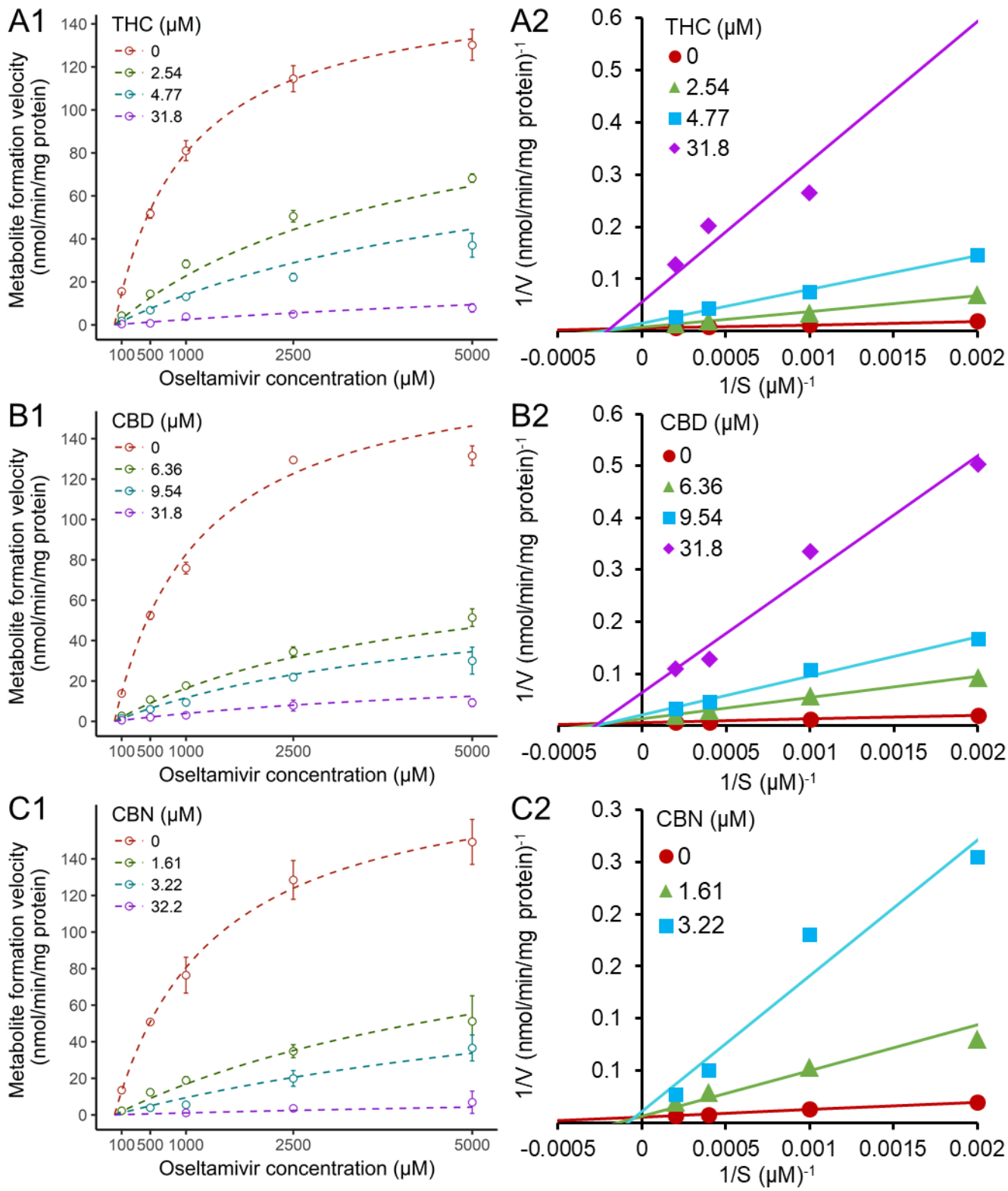

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Figure 7