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**Title:** Inhibition of tacrolimus metabolism by cannabidiol

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**Supplement**

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**Materials and Methods**

***Chemicals and Reagents***

Tacrolimus, rapamycin, and ketoconazole were purchased from MedChemExpress (Monmouth Junction, NJ). Cannabidiol, 7-hydroxy cannabidiol, and 7-carboxy cannabidiol were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (HLMs) from 50 donors of mixed gender and Cypex EasyCYPs Bactosomes (recombinant CYP3A4 and CYP3A5) were purchased from XenoTech (Kansas City, KS). NADPH was purchased from Dot Scientific Inc (Burton, MI). Sodium phosphate dibasic anhydrous (Na2H2PO4), sodium phosphate monobasic anhydrous (NaH2PO4), acetonitrile, methanol, methyl tertiary butyl ether (MTBE), and water were purchased from Fisher Scientific (Pittsburgh, PA). All chemicals and solvents were high-performance liquid chromatography (HPLC)-grade or higher.

***LC-MS/MS Method Development***

We developed methods for chromatographic separation and mass spectrometry analysis for tacrolimus and the internal standard (IS) rapamycin. Chromatographic separation was done using a ExionLC ™ AD Series Ultra-HPLC (UHPLC; ABSciex, Framingham, MA) equipped with two AD pumps, an AD autosampler, an AD column oven, and controller. It was couple to an QTRAP ® 6500+ mass spectrometer (ABSciex, Framingham, MA) fitted with an electrospray ionization source (ESI). The data was acquired using Analyst software 1.7.0 (ABSciex, Framingham, MA) with MultiQuant software 3.0.2 (ABSciex, Framingham, MA) on Windows 7®.

All method development, preliminary experiments, and tacrolimus depletion experiments were analyzed with the aforementioned UHPLC and mass spectrometer. Chromatographic separation was achieved using an Agilent Zorbax-SB C8 Rapid Resolution threaded column (150 x 4.6 mm inner diameter.; 5-μm particle size; Santa Clara, CA) on the UHPLC. An isocratic mobile phase composed of acetonitrile and 5 mM ammonium acetate (90:10, v/v) with a flow rate of 0.9 mL/min was used. The mass spectrometer was operated with ESI in the positive mode with multiple reaction monitoring (MRM). Global parameters for the mass spectrometer were optimized across all analytes under unit resolution for quadrupole 1 and 3: collision gas medium, curtain gas 30 psi, ion spray voltage 5500 V, source temperature 450°C, Gas 1 40 psi, Gas 2 50 psi, and ihe on. Tacrolimus and rapamycin with precursor-to-product ion transitions at m/z 821.3/768.3 and m/z 932.51/865.4 (ammonium adduct), respectively, were quantified with MRM. Their declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were as follows in respective order: (tacrolimus) 70, 10, 31, and 35; (rapamycin) 25, 10, 24, and 20.

We were unable to obtain major tacrolimus metabolites through a reliable commercial source, so the substrate depletion approach was chosen over the metabolite formation approach. Prior to each experiment, a linear standard curve of tacrolimus was generated. The concentrations to generate a standard were 0, 0.03, 0.1, 0.3, 1, 3, and 10 μM for incubation with HLMs, and 0, 0.03, 0.1, 0.3, 1, and 3 μM for incubation with rCYP3A4 and rCYP3A5. The lower limit of quantification (LLOQ) was 0.124 nM for both incubation systems. Quality control was performed at 0.02, 0.2, and 2 μM. Variabilities of standard and quality control samples were assessed by MultiQuant software. The acceptable intra-day and inter-day variabilities were less than 10% and 20%, respectively. The peak ratio of analytes was the same after 24 hours of the first LC-MS/MS analysis.

***Incubation procedures***

A total of five conditions were experimented to test our hypothesis – tacrolimus alone, tacrolimus with CBD, tacrolimus with 7-OH CBD, tacrolimus with 7-COOH CBD, and tacrolimus with ketoconazole as the positive control. These conditions were tested in HLM, rCYP3A4, and rCYP3A5. The experiment with HLM was performed in four technical replicates, whereas that with rCYP3A4 and rCYP3A5 were performed in two technical replicates.

Experiment 1 (pooled HLM)

The procedure for the incubation with HLMs was as follows. Tacrolimus stock solution was diluted with phosphate buffer (0.2 M; pH 7.4) to 1 μM in 0.65-mL tubes on a 96-well plate on ice. Into the dilutions, CBD (10 μM), 7-OH CBD (10 μM), 7-COOH CBD (10 μM), or ketoconazole (1 μM) was added. The resultant drug mixture should be 100 μL in each tube. To each tube, 30 μL of pooled HLMs (0.5 mg/mL final concentration) prepared in phosphate buffer was added. The 0.65-mL tubes were pre-warmed on a heating block at 37°C for 5 minutes and awaiting incubation. Finally, 20 μL of NADPH (1 mM) prepared in phosphate buffer was added to initiate the reaction. The reaction was quenched by adding 300 μL of ice-cold methanol with 10 ng/mL rapamycin at 0, 5, 10, 15, 20, and 30 minutes. The entire 96-well plate was vortexed for 30 seconds. The samples were transferred from 0.65-mL tubes to 16x100 mm disposable culture tubes with the addition of 2 mL of methyl tertiary butyl ether (MTBE). The plate with culture tubes was vortexed for 30 seconds and spun at 3600 RPM for 10 minutes. The supernatant was transferred to 13x100 glass test tubes at which the solvent was evaporated. The pellet was reconstituted with 60 μL of methanol, and 5 μL of it was injected into the mass spectrometer. Before and after each injection, the needle was washed with a mixture consisted of 50% of 0.1% formic acid in water, 25% of acetonitrile, and 25% of isopropanol.

Experiment 2 (rCYP3A4 & rCYP3A5)

The procedure for the incubation with rCYP3A4 and rCYP3A5 was slightly different. Tacrolimus stock solution was diluted with phosphate buffer (0.2 M; pH 7.4) to 1 μM. Rather than using 30 μL of HLMs, 10 μL of recombinant enzymes (20 pmol/150 μL final concentration) were used with 20 μL phosphate buffer to make up the remaining volume. In addition, the time points for adding ice-cold methanol and rapamycin to quench the reaction were 0, 1, 2.5, 5, 7.5, and 10 minutes.

***Kinetic analysis***

The amount of tacrolimus and rapamycin in analytes at each time point were displayed as chromatographic peaks on an absorbance-time plot. The area under the curve (AUC) of the peaks were obtained. The ratio of AUC for tacrolimus to rapamycin was calculated. The AUC ratio was converted to concentration of tacrolimus (μM) using the slope of the standard curve generated for each experiment. The concentration of tacrolimus decreased over time as it was metabolized by HLM, rCYP3A4, and rCYP3A5. As such, a plot of percent remaining of tacrolimus against time would show the rate of depletion of tacrolimus alone and when co-incubated with CBD, 7-OH CBD, 7-COOH CBD, or ketoconazole. The absolute value of the slope of the linear regression line is the first-order depletion rate constant from which the half-life was calculated using Equation (1):

(1)

where t1/2 is the half-life of substrate (min); kdep is the depletion rate constant (min-1).

***In Vitro-In Vivo Extrapolation (IVIVE)***

Experiment 1 (HLM)

The IVIVE of intrinsic clearance obtained from incubation with pooled HLMs was performed with a series of calculations.1 The *in vitro* intrinsic clearance (CLint,HLM) was calculated using Equation (2), where V (mL/mg) is the inverse of the concentration of microsomal protein; fumic,HLM is the non-specific binding to HLMs, which was estimated with the partition coefficient (logP) and concentration of microsomal protein ([HLM]) using Equation (3).2,3 From CLint,HLM, the *in vivo* intrinsic clearance of the liver (CLint) was extrapolated with two physiologically based scaling factors – microsomal protein per gram of liver (MPPGL) and liver weight/body weight (LW/BW) – using Equation (4). Further, the hepatic intrinsic clearance based on whole blood unbound drug concentration (CLH,B) was extrapolated from CLint,HLM by accounting for additional scaling factors – hepatic blood flow per body weight (QH) and fraction of unbound drug in blood (fuB) – using Equation (5). Hepatic blood flow was set at 24.5% of the average cardiac output of 5600 mL. 4,5 The factor fuB was calculated by dividing fu by blood-to-plasma ratio (B/P) as shown in Equation (6).6,7 Finally, hepatic extraction ratio derived from unbound drug concentration in whole blood (EH,B) were calculated using Equation (7).

(2)

(3)

(4)

(5)

(6)

(7)

*Experiment 2 (rCYP3A4 & rCYP3A5)*

The intrinsic clearance derived from recombinant enzymes (CLint,rCYPi) was calculated with Equation (8). The non-specific microsomal binding to rCYPi was corrected with Equation (9).

(8)

(9)

***Statistical Analysis***

All statistical analyses and data visualizations were performed with GraphPad Prism 10.2.0 (GraphPad software, Boston, MA) on Windows 10®. Descriptive statistics (mean and standard deviation) were obtained for percent tacrolimus remaining. Linear regression analysis was performed to fit the data for percent tacrolimus remaining on a semi-log scale. Data normality and equality of variance were assessed by Shapiro-Wilk test and F-test, respectively. Since the data followed Gaussian distribution with unequal variance, Brown-Forsythe and Welch ANOVA with post-hoc Dunnett’s T3 multiple comparison testing were used to compare the regression slopes of the control group to each of other groups. A two-tailed multiplicity adjusted p-value ≤ 0.05 was considered statistically significant.

**Table S1**

|  |  |  |
| --- | --- | --- |
| Parameters | Value | Source |
| Tacrolimus | | |
| logP | 3.26 | (Gertz et al, 2011)8 |
| fuP | 0.012 ± 0.0012 | (Zahir et al, 2001)9 |
| B/P ratio | 35 | (Zahir et al, 2001)9 |
| Physiological | | |
| MPPGL | 40 mg | (Hakooz et al, 2006)10 |
| LW/BW (Based on a 70-kg human) | 25.7 g/kg | (Davies and Morries, 1993)4 |
| QH,B (Based on a 70-kg human) | 19.6 mL/min/kg | (Davies and Morries, 1993)4 |

**Parameters for pharmacokinetic calculation and extrapolation.** B/P ratio, blood-to-plasma ratio; fuP, fraction unbound in plasma; logP, partition coefficient between octanol and water; LW/BW, liver weight per body weight; MPPGL, microsomal protein per gram of liver; QH,B, hepatic blood flow per body weight.

**Table S2**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Tacrolimus | Tacrolimus +  Ketoconazole | Tacrolimus +  CBD | Tacrolimus +  7-OH CBD | Tacrolimus +  7-COOH CBD |
| HLM (fumic,HLM = 0.672) |  |  |  |  |  |
| kdep (min-1) | 0.273 (0.249-0.297) | 0.024 (0.019-0.030) | 0.152 (0.138-0.167) | 0.083 (0.074-0.091) | 0.221 (0.195-0.248) |
| t1/2 (min) | 2.54 | 28.4 | 4.56 | 8.39 | 3.13 |
| Fold change of t1/2 | NA | 11.2 | 1.79 | 3.30 | 1.23 |
| CLint,HLM (mL/min/mg microsomal protein) | 0.812 | 0.073 | 0.452 | 0.246 | 0.65 |
| CLint (mL/min/kg) | 835 | 74.7 | 465 | 253 | 678 |
| CLH,B (mL/min) | 0.282 | 0.026 | 0.163 | 0.086 | 0.230 |
| EH,B | 0.014 | 0.001 | 0.008 | 0.004 | 0.012 |

**Pharmacokinetic parameters of tacrolimus depletion with pooled HLMs.** CBD, cannabidiol; CLH,B, hepatic intrinsic clearance of tacrolimus based on blood concentrations; CLint, *in vivo* intrinsic clearance of tacrolimus in a liver; CLint,HLM, *in vitro* intrinsic clearance of tacrolimus in human liver microsomes; EH,B, hepatic extraction ratio of tacrolimus based on blood concentrations; kdep; t1/2, half-life; 7-COOH CBD, 7-carboxycannabidiol; 7-OH CBD, 7-hydroxycannabidiol.

**Table S2**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Tacrolimus | Tacrolimus +  Ketoconazole | Tacrolimus +  CBD | Tacrolimus +  7-OH CBD | Tacrolimus +  7-COOH CBD |
| rCYP3A4 (fumic,rCYP3A4 = 0.885) | | | | | |
| kdep (min-1) | 0.752 (0.650-0.854) | 0.086 (0.049-0.123) | 0.114 (0.076-0.151) | 0.050 (0.015-0.084) | 0.659 (0.563-0.755) |
| t1/2 (min) | 0.922 | 8.06 | 6.11 | 14.0 | 1.05 |
| Fold change of t1/2 | NA | 8.74 | 6.63 | 15.2 | 1.14 |
| CLint,rCYP3A4 (μL/min/pmol rCYP3A4) | 6.37 | 0.729 | 0.962 | 0.420 | 5.59 |
| rCYP3A5 (fumic,rCYP3A5 = 0.885) | | | | | |
| kdep (min-1) | 1.97 (1.32-2.62) | 0.720 (0.615-0.826) | 0.065 (0.047-0.084) | 0.095 (0.056-0.134) | 1.75 (1.36-2.13) |
| t1/2 (min) | 0.351 | 0.962 | 10.6 | 7.30 | 0.397 |
| Fold change of t1/2 | NA | 2.74 | 30.3 | 20.8 | 1.13 |
| CLint,rCYP3A5 (μL/min/pmol rCYP3A5) | 16.7 | 6.11 | 0.552 | 0.804 | 14.8 |

**Pharmacokinetic parameters of tacrolimus depletion with rCYP3A4 and rCYP3A5.** CBD, cannabidiol; CLint,rCYP3A4, *in vitro* intrinsic clearance of tacrolimus in recombinant CYP3A4 enzymes; CLint,rCYP3A5, *in vitro* intrinsic clearance of tacrolimus in recombinant CYP3A5 enzymes; kdep, depletion rate constant; t1/2, half-life; 7-COOH CBD, 7-carboxycannabidiol; 7-OH CBD, 7-hydroxycannabidiol.

**Table S3**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Time (min) | Tacrolimus | Tacrolimus + Ketoconazole | Tacrolimus + CBD | Tacrolimus + 7-OH CBD | Tacrolimus + 7-COOH CBD |
| 0 | 100 | 100 | 100 | 100 | 100 |
| 5 | 48.8 ± 3.22 | 95.2 ± 15.0 | 65.0 ± 11.4 | 64.6 ± 6.46 | 55.3 ± 7.98 |
| 10 | 15.4 ± 3.30 | 88.5 ± 8.47 | 30.6 ± 4.73 | 42.3 ± 5.85 | 19.3 ± 5.72 |
| 15 | 2.18 ± 0.379 | 75.3 ± 7.97 | 12.5 ± 2.54 | 24.9 ± 8.49 | 5.64 ± 4.07 |
| 20 | 0.537 ± 0.173 | 61.0 ± 9.72 | 5.26 ± 1.39 | 16.5 ± 4.13 | 1.40 ± 0.410 |
| 30 | 0.481 ± 0.229 | 51.3 ± 8.49 | 3.44 ± 0.552 | 9.06 ± 1.54 | 0.513 ± 0.165 |

Descriptive statistics of percent tacrolimus remaining with pooled HLMs. Data are presented in mean ± SD (%).

**Table S4**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Time (min) | Tacrolimus | Tacrolimus + Ketoconazole | Tacrolimus + CBD | Tacrolimus + 7-OH CBD | Tacrolimus + 7-COOH CBD |
| 0 | 100 | 100 | 100 | 100 | 100 |
| 1 | 81.8 ± 64.3 | 107 ± 27.8 | 82.9 ± 23.4 | 75.4 ± 0.998 | 42.9 ± 9.44 |
| 2.5 | 27.1 ± 20.0 | 104 ± 9.87 | 53.9 ± 9.84 | 79.7 ± 9.39 | 12.0 ± 5.38 |
| 5 | 4.91 ± 4.71 | 77.4 ± 25.8 | 47.4 ± 9.16 | 67.9 ± 12.2 | 2.26 ± 0.004 |
| 7.5 | 0.413 ± 0.062 | 68.2 ± 20.7 | 30.4 ± 0.627 | 59.8 ± 12.0 | 0.768 ± 0.350 |
| 10 | 0.068 ± 0.009 | 42.7 ± 2.82 | 33.6 ± 1.24 | 59.9 ± 26.2 | 1.07 ± 0.253 |

Descriptive statistics of percent tacrolimus remaining with rCYP3A4. Data are presented in mean ± SD (%).

**Table S5**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Time (min) | Tacrolimus | Tacrolimus + Ketoconazole | Tacrolimus + CBD | Tacrolimus + 7-OH CBD | Tacrolimus + 7-COOH CBD |
| 0 | 100 | 100 | 100 | 100 | 100 |
| 1 | 5.47 ± 2.01 | 32.6 ± 3.17 | 75.0 ± 7.77 | 67.5 ± 13.7 | 10.4 ± 1.36 |
| 2.5 | 0.661 ± 0.236 | 7.80 ± 0.728 | 69.5 ± 6.00 | 53.3 ± 2.54 | 1.23 ± 0.471 |
| 5 | 0.254 ± 0.014 | 1.36 ± 0.518 | 60.9 ± 0.701 | 45.2 ± 10.1 | 0.530 ± 0.019 |
| 7.5 | 0.238 ± 0.064 | 0.441 ± 0.034 | 51.2 ± 2.32 | 36.1 ± 7.93 | 0.390 ± 0.222 |
| 10 | 0.370 ± 0.333 | 0.327 ± 0.059 | 48.4 ± 4.54 | 36.0 ± 9.84 | 0.908 ± 0.597 |

Descriptive statistics of percent tacrolimus remaining with rCYP3A5. Data are presented in mean ± SD (%).

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