Characterization of P-glycoprotein inhibition by major cannabinoids from marijuana

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P-gp, P-glycoprotein; THC, Δ⁹-tetrahydrocannabinol; THC-COOH, 11-Nor-Δ⁹-tetrahydrocannabinol-carboxylic acid; CBN, cannabinol; CBD, cannabidiol; VER, verapamil; Rh123, rhodamine123; DOX, doxorubicin, Caco-2, human adenocarcinoma cells; RBMECs, rat brain microvessel endothelial cells

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ABSTRACT

The ATP-dependent drug efflux transporter P-glycoprotein (P-gp) plays a significant role in the absorption and disposition of many compounds. The purpose of this study was to investigate the possible interaction of P-gp with each of four major marijuana constituents: Δ⁹-tetrahydrocannabinol (THC); 11-Nor-Δ⁹-tetrahydrocannabinol-carboxylic acid (THC-COOH); cannabinol (CBN) and cannabidiol (CBD). The results of a P-gp ATPase activity screen showed that THC-COOH, CBN, THC and CBD all stimulated P-gp ATPase activity with a Michaelis-Menten parameter (Vmax/Km) value of 1.3, 0.7, 0.1 and 0.05, respectively. Furthermore, CBD showed a concentration-dependent inhibitory effect on verapamil stimulated ATPase activity with an IC₅₀ value of 39.6 µM, while all other tested cannabinoids did not display appreciable inhibitory effects. Thus, the inhibitory effects of CBD on P-gp transport were further studied. At concentrations ranging from 5 µM to 100 µM, CBD robustly enhanced the intracellular accumulation of known P-gp substrates rhodamine123 and doxorubicin in a concentration dependent manner in Caco-2 and LLC-PK1/MDR1 cells. An IC₅₀ value of 8.44 µM was obtained for inhibition of P-gp function in LLC-PK1/MDR1 cells as determined by flow cytometry using rhodamine123 as a fluorescence probe. Following exposure to 30 µM CBD, the apparent permeability coefficient of rhodamine123 across Caco-2 and rat brain microvessel endothelial cell monolayers was increased to 2.2 and 2.6-fold in the apical to basolateral direction, but decreased to 0.69 and 0.47-fold in the basolateral to apical direction, respectively. These findings indicate that CBD significantly inhibits P-gp-mediated drug transport, suggesting CBD could potentially influence the absorption and disposition of other coadministered compounds that are P-gp substrates.
Introduction

P-glycoprotein (P-gp) is an ATP-dependent efflux transporter coded by the MDR1 (ABCB1) gene. It is expressed in multi-drug resistance tumor cells (Kartner et al., 1983), and in many normal tissues, including intestinal epithelium, hepatocytes, placenta, renal tubular cells and blood-brain barrier (BBB) (Cordon-Cardo et al., 1989; Lieberman et al., 1989; Fardel et al., 1993; Gatmaitan and Arias, 1993; Bendayan et al., 2002). It is widely acknowledged to play an important role in the disposition of many endogenous and exogenous chemicals. Human polymorphisms of the MDR1 gene can alter P-gp expression and function and result in altered drug pharmacokinetics and pharmacodynamics (Fromm, 2002). P-gp efflux transport is one of the major mechanisms responsible for the low oral bioavailability and limited brain penetration of many therapeutic drugs. Co-administration of P-gp inhibitors, such as verapamil (VER), PSC833 and GF120918, can efficiently increase the systemic absorption and brain concentration of P-gp substrate drug, and result in enhanced therapeutic as well as adverse drug effects of clinically administrated compounds (Mayer et al., 1997; Bardelmeijer et al., 2000; Nakagami et al., 2005). Therefore, the role of the interaction of P-gp inhibitors and substrates on drug disposition and drug effects has become an increasing focus of pharmacological research.

Marijuana (Cannabis sativa) is the most commonly used illegal drug in the United States. It is estimated that approximately 10% of individuals who ever use marijuana become daily users, and lifetime prevalence rates of marijuana dependence have been approximated at 4% of the population (Compton et al., 2004). In addition to behavioral
and psychotropic effects, cannabinoids, the primary constituents of marijuana, also have documented therapeutic properties. It is widely believed that cannabinoids produce their pharmacological effects by binding to two specific plasma membrane G protein-coupled receptors, the CB1 receptor, expressed mainly in the brain and in some peripheral tissues, and the CB2 receptor, expressed only outside of the central nervous system (CNS) (Pertwee and Ross, 2002). In recent years, numerous basic and clinical studies have confirmed that some cannabinoids exhibit the potential for therapeutic applications, including but not limited to treatment of pain, nausea, vomiting, multiple sclerosis, epilepsy, cancer, anxiety and depression (Hampson et al., 2000; Robson, 2001; Tramer et al., 2001; Killestein et al., 2003; Massi et al., 2004). However, very little is known regarding the possible interactions between cannabinoids and the P-gp or other drug transporter. Such effects could be important for understanding both the psychoactive and potential therapeutic effects of cannabinoids.

Accordingly, we initially investigated the P-gp affinity of each of four major cannabinoids, Δ⁹-tetrahydrocannabinol (THC), 11-Nor-Δ⁹-tetrahydrocannabinol-carboxylic acid (THC-COOH), cannabinol (CBN) and cannabidiol (CBD) using the P-gp-dependent ATPase activity assay. Initial experiments showed that all these cannabinoids stimulated P-gp ATPase activity to some extent in a concentration dependent manner (Wang et al., 2004a). Furthermore, CBD was found to exhibit a potent inhibitory effect on the VER stimulated ATPase activity. This result suggested that CBD might have an inhibitory effect on P-gp activity. Therefore, three different P-gp expressed cells, Caco-2, rat brain microvessel endothelial cells (RBMECs) and LLC-PK1/MDR1 cells, were used to more fully characterize the effect of CBD on the transport function of
P-gp \textit{in vitro}.
Materials and Methods

Materials

Human P-gp membranes (5 mg/ml) prepared from baculovirus-infected insect cells were purchased from BD Gentest (Woburn, MA). The human colon adenocarcinoma cell line Caco-2 at passage 17 was purchased from American Type Culture Collection (Manassas, VA) and was used for experiments from passage 30 to 40. The pig kidney epithelial cell line LLC-PK1 and its P-gp overexpressing mutant LLC-PK1/MDR1 cells were kindly provided by Dr. Kari Kivistö (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany). Fetal bovine serum, trypsin and Dulbecco’s modified eagle medium (DMEM) containing 4500 mg/L glucose, 4 mM L-glutamine and sodium pyruvate were obtained from Hyclone Co. (Logan, UT), collagenase II was from Worthington Biochemical Co. (Lakewood, NJ), dispase was obtained from BD Bioscience (Bedford, MA). MEM Non-Essential Amino Acid Solution was from Stemcell Technologies Inc. (Vancouver, British Columbia, Canada). Dulbecco’s Phosphate-Buffered Saline (DPBS), penicillin and streptomycin were all purchased from Mediatech Inc. (Herndon, VA). CBD, THC, THC-COOH, CBN, VER, fluorescein, doxorubicin (DOX) and rhodamine123 (Rh123) were obtained from Sigma Co. (St. Louis, MO). PSC833 was a gift from Novartis Pharmaceuticals (Switzerland). All other drugs and reagents were of analytical grade and commercially available.

ATPase assay

The human P-gp containing membranes were used for the measurements of the ATPase activity of P-gp. P-gp-dependent ATPase activity was quantified by determining the
increased inorganic phosphate (P<sub>i</sub>) concentrations in the presence of 100 µM of ortho-
vanadate as described previously (Boulton et al., 2002). Briefly, THC, THC-COOH, 
CBN and CBD were prepared freshly in Tris-MES buffer (pH 6.8) and added to the 
reaction mixture containing 40 µg P-gp membranes and 4 mM Mg-ATP at the final 
concentrations of 1, 5, 10, 25, 75, 100 µM. The reaction was initiated by adding ATP 
solution to the mixture. After incubation at 37º C for 40 min, the reactions were 
terminated by the addition of 30 µl of ice-cold 10 % sodium dodecylsulfate solution 
containing 0.1 % Antifoam A. Finally, P<sub>i</sub> was assayed by the ultraviolet (UV) absorption 
of the P<sub>i</sub>-molybdate complex at 620 nm and the P<sub>i</sub> concentrations were calculated from an 
eight-point standard curve established from 0-150 nM P<sub>i</sub> standard solution. The K<sub>m</sub> and 
V<sub>max</sub> values were estimated by fitting P<sub>i</sub> versus each compound concentration to the 
Michaelis-Menten equation using GraphPad Prism 4 software.

Cell Cultures

Primary culture of rat brain microvessel endothelial cells

RBMECs were isolated according to the modified method of Abbott and coworkers 
(Abott et al., 1992). Briefly, cerebral cortex was obtained from 10-15 day-old rats and 
washed 3 times with ice-cold DPBS. After surface vessels and meninges were removed, 
cortex gray matter was minced and incubated at 37º C for 1 h in serum-free DMEM 
containing 0.1 % collagenase II/dispase and 20 U/ml Dnase I. After digestion, the 
enzyme mixture was triturated with a Pasteur pipette, then centrifuged at 800 g for 5 min. 
The resultant pellet was resuspended in DPBS containing 25 % bovine serum albumin 
(BSA) and centrifuged at 2000 g at 4º C for 10 min. Fat, cell debris, and myelin floating
on the BSA were discarded and the pellet containing microvessels was resuspended and incubated in serum-free DMEM containing 0.1% collagenase II at 37°C for 30 min for a second digestion that served to remove the microvessel basement membrane. Released endothelial cells were further purified by centrifugation over a 50% Percoll gradient. The cells were maintained in DMEM supplemented with 20% fetal bovine serum, penicillin 100 kU/L and streptomycin 100 mg/L.

For the transport experiments, RBMECs were applied to polyester transwell membranes (0.4 µm pore size; 1 cm² growth area; Corning Inc. Corning, NY) at a density of 1 × 10⁵/cm². Culture medium was changed every 3 days. Transepithelial electric resistance (TEER) of RBMECs monolayers was monitored before transport studies using an EVOMeter fitted with chopstick electrodes (World Precision Instruments, Sarasota, FL), and the monolayers with TEER of greater than 250 Ω·cm² were utilized for the transport studies.

**LLC-PK1, LLC-PK1/MDR1 and Caco-2 cells**

LLC-PK1 and Caco-2 cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum, 1% MEM nonessential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin in an atmosphere of 5% CO₂ and 95% relative humidity. LLC-PK1/MDR1 cells were cultured under the same conditions except 640 nM of vincristine was added to the culture medium to maintain P-gp expression (Smit et al., 1998).
For P-gp substrate intracellular accumulation experiments, cells were seeded at a density of $1 \times 10^5$ cells/mL/well in 24-well plates. Culture medium was replaced every 2 days until cells reached confluence.

For transport experiments, Caco-2 cells were seeded onto polyester membrane filters (0.4 µm pores, 4.7 cm² growth area, Corning Inc. Corning, NY) of Transwell inserts at a density of $1 \times 10^5$ cells/cm². Culture medium was likewise changed every 2 days. Monolayers were used approximately 20 days postseeding when TEER reached 500 Ω·cm².

**Intracellular P-gp Substrate Accumulation Studies**

Intracellular uptake of P-gp substrates Rh123 and DOX was measured to evaluate the P-gp activity in LLC-PK1/MDR1 and Caco-2 cells while LLC-PK1 was included as a negative control (van der Sandt et al., 2000). After reaching confluence, LLC-PK1, LLC-PK1/MDR1 and Caco-2 cells were preincubated at 37ºC for 30min with transport buffer (serum-free DMEM containing 25mM HEPES, pH 7.4). Next, 0.5 % DMSO (vehicle control), specific concentrations of CBD, VER or PSC833 were added, then 5 µM of Rh123 or 1 µM of DOX were added for an additional 90 min incubation. After incubation, the solutions were discarded, and the cells were washed three times with ice-cold DPBS and solubilized with 1 % Triton X-100. Fluorescence of Rh123 and DOX were measured using HPLC equipped with a fluorescence detector. The concentrations were determined from the fluorescence value by the construction of Rh123 and DOX standard curves. The amount of Rh123 or DOX in each sample was standardized with the protein content determined by a Lowry assay.
A flow cytometry assay was also used to evaluate the effect of CBD on the intracellular accumulation of Rh123 in LLC-PK1/MDR1 cells and is described in detail elsewhere (Zhu and Liu, 2003). Briefly, cells were digested, collected, and incubated at 37 °C with 5 µM Rh123 in the absence or presence of various concentrations of CBD, VER and PSC833 for 60 min. After incubation, the cells were washed twice with ice-cold DPBS, resuspended in 200 µl of DPBS and analyzed by flow cytometry (FACScan, Becton Dickinson, USA). Data were analyzed with CellQuest software (Becton Dickinson, USA). An IC50 value for each compound was calculated when the inhibitory potency of 5 µM PCS833 was defined as 100 %.

Rh123 Transport Studies

When RBMECs or Caco-2 cells reached confluence, the integrity of monolayers was checked by both TEER value and the transport rate of fluorescein, a paracellular transport marker (van Bree et al., 1988). The qualified monolayers were rinsed two times with DPBS and preincubated with transport buffer at 37°C for 30 min. After preincubation, 0.5 % DMSO, CBD or VER was loaded at both sides of the monolayers, then Rh123 (5 µM) was added into the basal side for the basal to apical (B-A) transport study or apical side for the apical to basal (A-B) transport study. At designated times, 150 µl samples were taken from the receiver compartment, and the same volume of receiver compartment solution was replaced immediately after each sampling. Concentrations of Rh123 were determined by HPLC. Apparent permeability coefficients, $P_{\text{app}}$ (cm/s) were calculated according to the following equation:

$$P_{\text{app}} = \frac{dQ}{dt} \times (C^{-1} A^{-1}) \quad (\text{Eq.1})$$
where \( dQ/dt \) is the rate at which the Rh123 appears in the receiver compartment, \( A \) is the diffusion area (cm\(^2\)), and \( C \) is the initial concentration of Rh123 in the donor compartment.

**HPLC Analysis**

HPLC assay was utilized for the quantitative analysis of Rh123 and DOX. The HPLC system consisted of a Waters 2690 Separations module (Waters, Milford, MA), a C18 reversed-phase column (300 \( \times \) 4.1 mm i.d., Versoack, Alltech, Deerfield, IL) and a Waters 474 scanning fluorescence detector (Waters, Milford, MA). The separation of Rh123 was carried out isocratically using 20 mM of sodium acetate buffer (pH 4.0): acetonitrile (40:60, v:v) at a flow rate of 1.0 mL/min. The mobile phase for DOX samples consisted of a 50 mM NaH\(_2\)PO\(_4\) buffer (pH 3.0): acetonitrile (69:31, v:v) mixture with a flow rate of 1.0 mL/min. The detection wavelengths were set at 488 nm (\( \lambda_{ex} \)), 535 nm (\( \lambda_{em} \)) for Rh123 and 480 nm (\( \lambda_{ex} \)), 560 nm (\( \lambda_{em} \)) for DOX, respectively.

**Statistical Analysis**

Statistical analysis for significant differences was performed using the two-tailed Student’s \( t \) test. A probability of <0.05 was considered to be statistically significant.
Results

Effects of Cannabinoids on the P-gp ATPase Activity

All four cannabinoids tested stimulated P-gp ATPase activity to some degree in a concentration dependent manner (Figure 1, Table 1). The rank order for stimulation of ATPase activity ($V_{\text{max}}/K_m$) was VER (3.8) > THC-COOH (1.3) > CBN (0.7) > THC (0.1) > CBD (0.05). The effect of the cannabinoids on inhibiting VER stimulated ATPase activity was also investigated by measuring the production of $P_i$ stimulated by 10 µM VER in the presence or absence of each cannabinoid. The data indicated that among the tested cannabinoids, only CBD exhibited concentration-dependent inhibitory effects on the VER stimulated P-gp ATPase activity with a calculated IC$_{50}$ value of 39.6 µM. All other cannabinoids did not show any appreciable inhibitory effects (Figure 2). Therefore, further cellular transport studies were focused only on CBD.

CBD Increased the Intracellular Accumulation of Rh123 and DOX in LLC-PK1/MDR1 and Caco-2 Cells

The inhibitory potential of CBD on the activity of P-gp efflux was tested in Caco-2 and LLC-PK1/MDR1 cells. CBD caused a concentration-dependent increase in the intracellular accumulation of both Rh123 and DOX in Caco-2 (Figure 3 A,B). The intracellular accumulation of Rh123, a classical P-gp fluorescence substrate, was enhanced 1.5 and 2.2-fold in the presence of 10 and 30 µM of CBD, respectively ($p<0.01$). The accumulation of DOX, another well-known P-gp substrate, was also increased to 1.6, 3.7 and 7.4-fold in the presence of CBD at the concentration of 3, 10 and
30 µM, respectively (p<0.01). The classical P-gp inhibitors, VER (30 µM) and PSC833 (1 µM), exhibited potent inhibitory effects on the P-gp efflux function and resulted in a significant increase of intracellular accumulation of both Rh123 and DOX in Caco-2 cells (p<0.01 versus control).

The human MDR1 cDNA-transfected cell line, LLC-PK1/MDR1, highly expresses functional P-gp and is widely utilized in P-gp studies (Evers et al., 1996; van Helvoort et al., 1996). Due to the P-gp efflux, much less intracellular retention of Rh123 and DOX were found in LLC-PK1/MDR1 cells than in LLC-PK1 cells. The intracellular accumulation of Rh123 in LLC-PK1/MDR1 cells was significantly increased by 20, 100 µM CBD, or 1 µM PSC833 (118.35 ± 3.33 %, 216.90 ± 24.40 %, and 258.69 ± 26.27 % of the control, respectively; p<0.01). A more potent P-gp reversal effect of CBD was found when DOX was used as the P-gp substrate. The accumulation of DOX in LLC-PK1/MDR1 cells was enhanced to 199.96 ± 22.15 %, 437.53 ± 64.72 %, 1420.97 ± 152.25 %, and 1166.59 ± 71.18 % of the control by 5, 20, 100 µM CBD, and 1 µM PSC833, respectively (p<0.01). As expected, in P-gp negative LLC-PK1 cells, no significant effects on the accumulation of Rh123 or DOX were observed in the presence of either CBD (100 µM) or PSC833 (1 µM) (Figure 4 A, B).

Flow cytometry experiments also indicated that CBD increased the intracellular accumulation of Rh123 by inhibiting P-gp function in a concentration dependent manner (Figure 5). The IC₅₀ values of CBD, VER and PSC833 were 8.44 ± 0.58, 6.99 ± 0.66 and 0.38 ± 0.03 µM, respectively.

**Effect of CBD on the Transport of Rh123 across Caco-2 and RBMECs Monolayers**
To investigate the possible influence of CBD on P-gp substrate transport across some areas of localized expression of P-gp, such as intestine and the BBB, Caco-2 and RBMECs monolayers were used for the studies of the transport of Rh123 in both B-A and A-B directions. As shown in Table 2, Rh123 exhibited highly polarized transport across Caco-2 cell and RBMECs monolayers (i.e., $P_{\text{app B-A}} \gg P_{\text{app A-B}}$). The $P_{\text{app B-A}}/P_{\text{app A-B}}$ ratio of Rh123 across Caco-2 and RBMECs monolayers was 33.72 and 12.10, respectively. The presence of CBD or VER significantly decreased the transport of Rh123 across Caco-2 monolayers in B-A direction but increased the Rh123 transport in the reverse direction (Figure 6). These data indicated that the treatments of 10 and 30 µM of CBD resulted in 25.1 % and 31.1 % decreases of Rh123 $P_{\text{app B-A}}$ values and 75.6 % and 116.7 % increases of Rh123 $P_{\text{app A-B}}$ values, respectively (Table 2; p<0.01).

Regarding RBMECs, the effects of CBD on the transport of Rh123 across the monolayers were similar to that observed in Caco-2 cells (Figure 7). After incubation for 120 min, a 52.5 % decrease of $P_{\text{app B-A}}$ value and a 160.6 % increase of $P_{\text{app A-B}}$ value compared to the vehicle control were observed in the presence of 30 µM of CBD (Table 2; p<0.01).
Discussion

The P-gp-dependent ATPase assay is a widely used \textit{in vitro} method to investigate the affinity of compounds for P-gp (Shapiro and Ling, 1994). It is unclear why some chemicals (VER, vinblastine, DOX) stimulate ATPase activity, while others (cyclosporin A, PSC833) inhibit both basal and VER stimulated ATPase activity. One possible explanation is that P-gp ATPase has catalytic and inhibitory sites and that the different affinities of various compounds for these two sites will determine whether P-gp ATPase is stimulated or inhibited (Dey et al., 1997). Whatever alteration of P-gp ATPase activity occurs (i.e. inhibition or stimulation), it indicates that the compound has affinity for P-gp, and is a potential substrate and/or inhibitor. In the present study, the ATPase assay was initially used to investigate the possible interaction between P-gp and four major constituents in marijuana: the principal psychoactive constituent, THC, and its metabolite THC-COOH; the major non-psychoactive constituent CBD and another major cannabinoid, CBN. The results indicated that all four compounds exhibited some degree of ATPase stimulation. Compared to our previous data testing various second-generation antipsychotic drugs (Boulton et al., 2002), THC-COOH ($V_{max}/K_m=1.3$) and CBN ($V_{max}/K_m=0.7$) have comparable ATPase activity with olanzapine ($V_{max}/K_m=0.8$). Olanzapine was later confirmed by our laboratory to be a P-gp substrate in \textit{in vivo} experiments utilizing P-gp knock out mice (mdr1a/1b (-/-) mice). In that study, transgenic mice exhibited a 3-fold higher brain to plasma concentration ratio of olanzapine than control animals (Wang et al., 2004b). Two other cannabinoids, THC and CBD, exhibited relatively lower ATPase activities with $V_{max}/K_m$ values of 0.1 and 0.05, respectively. In addition, no influences on VER stimulated ATPase activity were observed in the
presence of a series of concentrations of THC-COOH, CBN, and THC. These findings suggested that THC-COOH and CBN are likely P-gp substrates. These two compounds may bind to the catalytic sites of ATPase to stimulate ATPase activity. However, their affinities to P-gp seem to less than VER’s, since the ATPase activity stimulated by 10 \( \mu \text{M} \) VER were not altered with co-incubation of THC-COOH and CBN even at a concentration of 100 \( \mu \text{M} \). Interestingly, CBD had a potent inhibitory effect on the VER stimulated ATPase activity which occurred in a concentration dependent manner. Therefore, further \textit{in vitro} experiments were conducted in cell culture models to determine whether CBD is a P-gp inhibitor.

These data indicated that CBD significantly increased the intracellular accumulation of Rh123 and DOX in Caco-2 and LLC-PK1/MDR1 cells in a concentration dependent manner (Figures 3, 4). The P-gp inhibitory potency of CBD (IC\(_{50}=8.44 \pm 0.58 \mu \text{M}\)), determined by flow cytometry assay in LLC-PK1/MDR1 cells, was much lower than that of PSC833 (IC\(_{50}=0.38 \pm 0.03 \mu \text{M}\)), but comparable to that of another classic P-gp inhibitor, VER (IC\(_{50}=6.99 \pm 0.66 \mu \text{M}\)). It is interesting that CBD seems to have a stronger effect on increasing the intracellular accumulation of DOX than that of Rh123 in both cell lines studies. The increase in the intracellular accumulation of Rh123 and DOX in Caco-2 cells produced by 30 \( \mu \text{M} \) CBD was 106.6\% and 641.0\% of control, respectively, while in LLC-PK1/MDR1 cells, 100 \( \mu \text{M} \) CBD produced 116.8\% and 1321.0\% effects of control, respectively. The reason for this apparent substrate-dependent effect is a matter of speculation but could be related to different affinity of substrate and P-gp or caused by the participation of other transporters. Other investigators have reported that some influx transporters, including organic cation transporters (OCTs) which belong to solute carrier
family 22 (SLC22), are also expressed in Caco-2 and LLC-PK1/MDR1 cells and are involved in the transport of Rh123 (Masereeuw et al., 1997; van der Sandt et al., 2000). OCT mediated Rh123 influx may be one of the reasons why Rh123 exhibits less response for P-gp inhibitors than DOX.

A number of therapeutic drugs have been demonstrated to be P-gp substrates. Physiological expression of P-gp in tissues can be a major determinant of the disposition of these drugs and provides a cellular defense mechanism against potentially harmful compounds. Knock out mice devoid of P-gp display large changes in the pharmacokinetics and pharmacodynamics of many drugs that are P-gp substrates (Schinkel et al., 1995a; Schinkel et al., 1995b). Co-administration of P-gp inhibitors, such as PSC833, GF120918 and VER, can potently inhibit the function of P-gp in intestine and the BBB, and result in dramatic increases of oral absorption and brain entry of P-gp substrates (Mayer et al., 1997; Bardelmeijer et al., 2000; Nakagami et al., 2005). Therefore, there is increasing interest in the possible role of P-gp mediated drug-drug interactions on therapeutic drug efficacy and toxicity. It is well known that P-gp is highly expressed on the apical membrane of Caco-2 and RBMECs monolayers resulting in a directional transport of P-gp substrates, i.e. higher transport rate in the B-A direction and lower transport rate in the reverse direction (Cordon-Cardo et al., 1989; Bendayan et al., 2002). Thus, Caco-2 cells and RBMECs have been widely used as in vitro models of intestinal absorption and BBB transport. In this study, Caco-2 and RBMECs monolayers were used to evaluate the potential for influence of CBD on intestinal drug absorption and brain entry. As expected, the data showed that CBD significantly decreased the Rh123 transport in the B-A direction, whereas it increased the transport in the A-B
direction in both Caco-2 and RBMECs monolayers. The present results are consistent with the characterization of CBD as a potent P-gp inhibitor, and at sufficient concentrations CBD may significantly influence the absorption and disposition of drugs that are P-gp substrates. It has been reported that CBD pretreatment of mice increased brain levels of THC, cocaine and phencyclidine approximately 3-, 2- and 4-fold, respectively (Reid and Bornheim, 2001). Behavioral tests also indicated that the CBD-mediated increases in the brain concentrations of these drugs correlated with increased pharmacological responses (Reid and Bornheim, 2001). The underlying mechanism of this observation is not clear. However, the findings in the present study, generated from several experimental methods suggest that CBD is a potent P-gp inhibitor that can dramatically influence the transport of P-gp substrates in intestine and BBB cellular models. Therefore, it is possible that the increased THC brain concentration after pretreatment with CBD may be due to P-gp inhibitory effects on BBB and P-gp substrate status of THC.

One issue of importance when considering the possible influence of P-gp inhibitors on the pharmacokinetics of co-administered drugs, is its inhibitory potency and the concentration that the tested P-gp inhibitor can attain with clinically relevant dosing regimens or exposure in situations of substance abuse. As far as we know, the concentration of CBD achieved within human tissues which express P-gp (brain, liver, intestine, kidney, and placenta) remains unknown. The mean plasma concentration has been reported to range from 18.8 to 35.6 nM after daily oral doses of CBD 10 mg/kg per day for 6 weeks in patients with Huntington’s disease (Consroe et al., 1991). A recent study indicated that the mean maximum concentration is approximately 3.0 nM obtained
60 min after oral administration of cannabis extract containing 5.4 mg CBD (Nadulski et al., 2005). These concentrations are much lower than its in vitro effective concentration (~3 µM). However, when assessed for some other investigational uses such as potential anticancer activities, CBD may be given at a high dose and subsequently reach a higher concentration in vivo, since CBD can be safely administrated to humans in high doses without apparent toxicities (Grotenhermen, 2003). In addition, when given orally, CBD concentration in the gastrointestinal tract may reach a level high enough to produce a potent inhibitory effect on P-gp activity of intestinal epithelium and result in the enhanced bioavailability of P-gp substrates. Furthermore, since localized tissue accumulation of lipophilic compounds commonly occurs, CBD tissue concentration is expected to be higher than that in plasma. Up to now, no details regarding in vivo CBD concentration in marijuana abusers have been released. However, considering the frequently extensive, and long-term heavy use of marijuana, it is a reasonable assumption that CBD concentration may reach a much higher level in some heavy users than that in patients treated at typical investigational doses. Considering that VER, which exhibited similar P-gp inhibitory potency of CBD in our in vitro studies, can produce significant in vivo P-gp inhibitory effects, it is a reasonable proposition that CBD could exert significant inhibitory effects on P-gp in vivo and result in the alteration of pharmacokinetics and pharmacodynamics of other drugs. However this requires confirmation by further in vivo experiments in animal models.

CBD is the major non-psychoactive constituent in marijuana, and exhibits many pharmacological properties suggesting putative anticonvulsant, anxiolytic, antipsychotic, antinauseant, and antiinflammatory effects (Mechoulam et al., 2002). Due to its lack of
psychoactive activity, CBD may be a potential candidate for various clinical applications. Recent studies indicated that CBD can limit tumor growth in vitro and in vivo by inducing programmed cell death, and can also inhibit tumor migration through a cannabinoid receptor-independent mechanism (Massi et al., 2004; Vaccani et al., 2005). It is well known that P-gp related multidrug resistance (MDR) is one of the major problems associated with anticancer chemotherapy. One strategy for reversal of P-gp mediated MDR is combined use of anticancer drugs with P-gp inhibitors. The MDR reversal effect of P-gp inhibitors, VER, and PSC833 et al, have been proven in both laboratory and clinical studies (Advani et al., 2001; Baekelandt et al., 2001). Therefore, the P-gp reversal property of CBD could be a valuable compliment to its possible direct anticancer applications.

The molecular mechanism of P-gp inhibition by CBD cannot be clearly revealed from our present studies. However, since CBD significantly inhibits the ATPase activity stimulated by P-gp substrate VER, one hypothesis is that CBD binds to a specific site of P-gp to inhibit the activation of ATPase stimulated by P-gp substrates, and subsequently decreases the energy requirement for the transport of P-gp substrates, and results in the inhibition of P-gp function.

In summary, a variety of in vitro methodologies and cell lines were utilized to assess the interactions of P-gp and four major marijuana constituents, THC, THC-COOH, CBN and CBD. The data generated suggested that all four cannabinoids, especially THC-COOH and CBN, are potential P-gp substrates, which implies that their pharmacokinetics and pharmacodynamics may be affected by different gene types of MDR1 or co-
administration of P-gp inhibitors. Furthermore, experiments based on P-gp expressed cells, Caco-2, LLC-PK1/MDR1 and RBMECs, demonstrated that CBD exhibits a potent P-gp inhibitory effect, suggesting that CBD could potentially influence the absorption and disposition of other co-administered compounds that are P-gp substrates.
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Footnotes

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Legends For Figures

Figure 1. Concentration-dependence of ortho-vanadate sensitive substrate-induced P-gp ATPase activity as measured by inorganic phosphate release by THC, THC-COOH, CBN, CBD and VER.

Figure 2. The effects of THC, THC-COOH, CBN and CBD on the VER stimulated ATPase in P-gp expressed membranes. A fixed concentration of VER (10 µM) was incubated with different concentrations of cannabinoids. Values are the average of duplicate determinations.

Figure 3. Effect of CBD on the intracellular accumulation of Rh123 (A) and DOX (B) in Caco-2 cells. The intracellular accumulation of Rh123 (5 µM) and DOX (1 µM) in Caco-2 cells after 1 h incubation in the presence or absence of CBD (1, 3, 10, 30 µM), VER (30 µM), or PSC833 (1 µM) was determined as described in the methods section. Each data value is the mean with a bar representing SD, n=3. **p<0.01 versus control.

Figure 4. Effect of CBD on the intracellular accumulation of Rh123 (A) and DOX (B) in LLC-PK1 and LLC-PK1/MDR1 cells. Inhibitory effect of CBD on P-gp function in LLC-PK1/MDR1 cells was assessed by measuring the intracellular concentrations of Rh123 and DOX after incubation for 1 h in the presence or absence of CBD (5, 20, 100 µM). LLC-PK1 cells were also included for the negative control. Each data value is the mean with a bar representing SD, n=3. **p<0.01 versus control.

Figure 5. Comparison of the concentration-dependent P-gp inhibitory effect of CBD, VER and PSC833 by flow cytometry assay. Flow cytometry was used to measure the
accumulation of Rh123 in LLC-PK1/MDR1 cells after incubation with CBD (2, 5, 10, 20, 50, 100 µM), VER (2, 5, 10, 20, 50, 100 µM), or PSC833 (0.1, 0.2, 0.5, 1, 2, 5 µM) for 1 h. Inhibition of Rh123 efflux caused by the treatment of 5 µM PSC833 was defined as 100 % inhibition. Results are expressed as the mean with a bar representing SD, n=3.

**Figure 6.** Effect of CBD on the directional transport of Rh123 across Caco-2 cell monolayers. Rh123 (5 µM) was added to the basal (for B-A transport) or apical side (for A-B transport) of Caco-2 cell monolayers, while CBD (3, 10, 30 µM) or VER (100 µM) was added to both sides of monolayers. The amounts of Rh123 in the receiver compartment were determined at 15, 30, 60, 90, 120 min after incubation. Each point represents the mean ± SD of three experiments.

**Figure 7.** Effect of CBD on the directional transport of Rh123 across the RBMECs monolayers. Rh123 (5 µM) was added to the donor compartment, while CBD (30 µM) or VER (100 µM) was added to both the apical and basal compartment. After 120 min incubation, concentration of Rh123 in receiver compartment was determined to assess inhibition of P-gp mediated Rh123 transport by CBD and VER. Data are expressed as percentage of the transport of Rh123 in B-A direction of control (n=3). **p<0.01 versus B-A control. ##p<0.01 versus A-B control.
Table 1. The $K_m$, $V_{max}$, and $Cl_{int}$ ($V_{max}/K_m$) values of cannabinoids on ortho-vanadate-sensitive ATPase activity in expressed P-gp membranes (data were mean of duplicate determinations)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
<th>$Cl_{int}$ (min$^{-1} \times 10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>5.2 ± 0.9</td>
<td>19.62 ± 0.71</td>
<td>3.8</td>
</tr>
<tr>
<td>THC-COOH</td>
<td>5.1 ± 3.4</td>
<td>6.5 ± 0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>CBN</td>
<td>3.9 ± 4.9</td>
<td>2.6 ± 0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>THC</td>
<td>49.1 ± 27.7</td>
<td>5.4 ± 1.4</td>
<td>0.1</td>
</tr>
<tr>
<td>CBD</td>
<td>20.4 ± 38.4</td>
<td>1.1 ± 0.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 2. Effect of CBD on Apparent Permeability Coefficients ($P_{app}$) of Rh123 (5 µM) across Caco-2 and RBMECs monolayers

<table>
<thead>
<tr>
<th>Cell</th>
<th>Inhibitor</th>
<th>$P_{app}(\text{cm s}^{-1} \times 10^7)$</th>
<th>Ratio ($P_{app \text{B-A}}/P_{app \text{A-B}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B-A</td>
<td>A-B</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>26.30 ± 1.41</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td>Caco-2</td>
<td>3 µM CBD</td>
<td>24.68 ± 4.75</td>
<td>1.02 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>10 µM CBD</td>
<td>19.69 ± 0.92**</td>
<td>1.37 ± 0.38**</td>
</tr>
<tr>
<td></td>
<td>30 µM CBD</td>
<td>18.13 ± 1.99**</td>
<td>1.69 ± 0.19**</td>
</tr>
<tr>
<td></td>
<td>100 µM VER</td>
<td>4.92 ± 0.57**</td>
<td>1.92 ± 0.50**</td>
</tr>
<tr>
<td>RBMECs</td>
<td>Vehicle</td>
<td>11.98 ± 0.30</td>
<td>0.99 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>30 µM CBD</td>
<td>5.69 ± 0.23**</td>
<td>2.58 ± 0.19**</td>
</tr>
<tr>
<td></td>
<td>100 µM VER</td>
<td>4.14 ± 0.14**</td>
<td>3.24 ± 0.10**</td>
</tr>
</tbody>
</table>

Results are reported as mean ± SD, n=3. *p<0.05, **p<0.01 versus vehicle control.
Figure 1
Figure 2

![Graph showing drug concentration vs. % of control activity for THC, THC-COOH, CBN, and CBD]
Figure 3A

Accumulation of Rh123 (nmol/mg protein)

Control  1µM  3µM  10µM  30µM  VER 30µM  PSC833 1µM

A

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JPET Fast Forward. Published on January 26, 2006 as DOI: 10.1124/jpet.105.098541
Figure 3B

Accumulation of DOX (nmol/mg protein)

Control          1µM        3µM          10µM        30µM         VER 30µM    PSC833 1µM

**

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Accumulation of Rh123 (nmol/mg protein)

**Figure 4A**

A

LLC-PK1/MDR1

Control 5µM 20µM 100µM PSC833 1µM

LLC-PK1

Control CBD 100µM PSC833 1µM

CBD
Accumulation of DOX
(nmol/mg protein)

Control 5µM 20µM 100µM PSC833 1µM  Control CBD 100µM PSC833 1µM

LLC-PK1/MDR1

LLC-PK1

Figure 4B
Figure 5

Concentration (µM)

Inhibition of P-gp efflux function

Inhibitory effect induced by 5µM PSC833 is defined as 100%.

Concentration (µM)
Figure 6

B-A

A-B
Rh123 transport (% of control in B-A direction)

Control    CBD 30µM   VER 100µM

Control    CBD 30µM   VER 100µM

B-A

A-B

Figure 7