

Characterization of P-glycoprotein Inhibition by Major Cannabinoids from Marijuana

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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: Δ^9 -tetrahydrocannabinol (THC), 11-nor- Δ^9 -tetrahydrocannabinol-carboxylic acid (THC-COOH), cannabino- (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 (V_{max}/K_m) 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 IC_{50} value of 39.6 μ M, whereas 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 to 100 μ M, CBD robustly en-

hanced the intracellular accumulation of known P-gp substrates rhodamine 123 and doxorubicin in a concentration-dependent manner in Caco-2 and LLC-PK1/MDR1 cells. An IC_{50} value of 8.44 μ M was obtained for inhibition of P-gp function in LLC-PK1/MDR1 cells as determined by flow cytometry using rhodamine 123 as a fluorescence probe. Following exposure to 30 μ M CBD, the apparent permeability coefficient of rhodamine 123 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.

P-glycoprotein (P-gp) is an ATP-dependent efflux transporter coded by the multidrug resistance (MDR)1 (ABCB1) gene. It is expressed in multidrug 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. Coadministration of P-gp inhibitors, such as verapamil (VER), valsopodar (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 administered 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

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ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; VER, verapamil; BBB, blood-brain barrier; THC, Δ^9 -tetrahydrocannabinol; THC-COOH, 11-nor- Δ^9 -tetrahydrocannabinol-carboxylic acid; CBN, cannabino-; CBD, cannabidiol; RBMEC, rat brain microvessel endothelial cell; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; DOX, doxorubicin; Rh123, rhodamine 123; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; TEER, transepithelial electric resistance; HPLC, high-performance liquid chromatography; B-A, basal-to-apical; A-B, apical-to-basal.

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 held 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 (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— Δ^9 -tetrahydrocannabinol (THC), 11-nor- Δ^9 -tetrahydrocannabinol-carboxylic acid (THC-COOH), cannabinal (CBN), and cannabidiol (CBD)—using the P-gp-dependent ATPase activity assay. Initial experiments showed that all of 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 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's medium (DMEM) containing 4500 mg/l glucose, 4 mM L-glutamine, and sodium pyruvate were obtained from Hyclone Laboratories (Logan, UT); collagenase II was from Worthington Biochemical (Lakewood, NJ); and Dispase was obtained from BD Biosciences (Bedford, MA). MEM nonessential amino acid solution was from Stemcell Technologies Inc. (Vancouver, BC, Canada). Dulbecco's phosphate-buffered saline (DPBS), penicillin, and streptomycin were all purchased from Mediatech (Herndon, VA). CBD, THC, THC-COOH, CBN, VER, fluorescein, doxorubicin (DOX), and rhodamine 123 (Rh123) were obtained from Sigma-Aldrich (St. Louis, MO). PSC833 was a gift from Novartis Pharmaceuticals (Basel, 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_i) concentrations in the presence of 100 μ M *ortho*-vanadate as described previously (Boulton et al., 2002). In brief, THC, THC-COOH, CBN, and CBD were prepared freshly in Tris-2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.8, and added to the reaction mixture containing 40 μ g of P-gp membranes and 4 mM Mg-ATP at the final concentrations of 1, 5, 10, 25, 75, and 100 μ M. The reaction was initiated by adding ATP solution to the mixture. After incubation at 37°C for 40 min, the reactions were terminated by the addition of 30 μ l of ice-cold 10% sodium dodecylsulfate solution containing 0.1% Antifoam A. Finally, P_i was assayed by the UV absorption of the P_i -molybdate complex at 620 nm, and the P_i concentrations were calculated from an eight-point standard curve established from 0 to 150 nM P_i standard solution. The K_m and V_{max} values were estimated by fitting P_i versus each compound concentration to the Michaelis-Menten equation using Prism 4 software (GraphPad Software Inc., San Diego, CA).

Cell Cultures

Primary Culture of Rat Brain Microvessel Endothelial Cells. RBMECs were isolated according to the modified method of Abbott et al. (1992). In brief, cerebral cortex was obtained from 10- to 15-day-old rats and washed three times with ice-cold DPBS. After surface vessels and meninges were removed, cortex gray matter was minced and incubated at 37°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 and then centrifuged at 800g for 5 min. The resultant pellet was resuspended in DPBS containing 25% bovine serum albumin and centrifuged at 2000g at 4°C for 10 min. Fat, cell debris, and myelin floating on the bovine serum albumin 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, 100 kU/l penicillin, and 100 mg/l streptomycin.

For the transport experiments, RBMECs were applied to polyester Transwell membranes (0.4- μ m pore size; 1-cm² growth area; Corning Glassworks, Corning, NY) at a density of 1×10^5 /cm². Culture medium was changed every 3 days. Transepithelial electric resistance (TEER) of RBMEC monolayers was monitored before transport studies using an EVOMeter fitted with chopstick electrodes (WPI, Sarasota, FL), and the monolayers with TEER of greater than 250 Ω /cm² were used 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 μ g/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 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 1×10^5 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- μ m pores, 4.7-cm² growth area; Corning Glassworks) of Transwell inserts at a density of 1×10^5 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, whereas 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 37°C for 30 min with transport buffer (serum-free DMEM containing 25 mM HEPES, pH 7.4). Next, 0.5% dimethyl sulfoxide (vehicle control), or specific concentrations of CBD, VER, or PSC833 were added, and then 5 μM Rh123 or 1 μM DOX was 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 was described in detail previously (Zhu and Liu, 2003). In brief, 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, San Jose, CA). Data were analyzed with CellQuest software (Becton Dickinson). An IC_{50} value for each compound was calculated when the inhibitory potency of 5 μM PSC833 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, and then 5 μM Rh123 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_{app} (centimeters per second) were calculated according to the following equation: $P_{\text{app}} = (dQ/dt) \times (C^{-1}A^{-1})$, where dQ/dt is the rate at which the Rh123 shows up in the receiver compartment, A is the diffusion area (square centimeters), and C is the initial concentration of Rh123 in the donor compartment.

HPLC Analysis

HPLC assay was used for the quantitative analysis of Rh123 and DOX. The HPLC system consisted of a Waters 2690 Separations

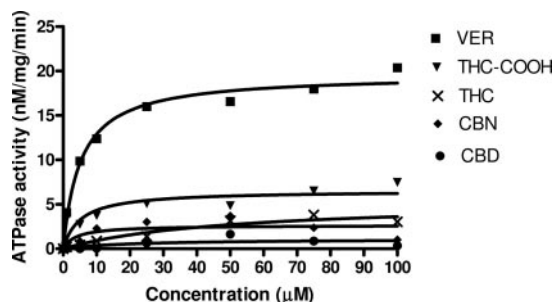


Fig. 1. Concentration dependence of *ortho*-vanadate-sensitive substrate-induced P-gp ATPase activity as measured by inorganic phosphate release by THC, THC-COOH, CBN, CBD, and VER.

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. The separation of Rh123 was carried out isocratically using 20 mM 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_2PO_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 (λ excitation) and 535 nm (λ emission) for Rh123 and at 480 (λ excitation) and 560 nm (λ emission) 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 (Fig. 1; Table 1). The rank order for stimulation of ATPase activity (V_{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 (Fig. 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 (Fig. 3, A and B). The intracellular accumulation of Rh123, a classic P-gp fluorescence substrate, was enhanced 1.5- and 2.2-fold in the presence of 10 and 30 μM 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 classic 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 used in

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 are mean of duplicate determinations.

	K_m	V_{max}	Cl_{int}
	μM	nmol/mg/min	$\text{min}^{-1} \times 10^{-3}$
Verapamil	5.2 \pm 0.9	19.62 \pm 0.71	3.8
THC-COOH	5.1 \pm 3.4	6.5 \pm 0.9	1.3
CBN	3.9 \pm 4.9	2.6 \pm 0.6	0.7
THC	49.1 \pm 27.7	5.4 \pm 1.4	0.1
CBD	20.4 \pm 38.4	1.1 \pm 0.6	0.05

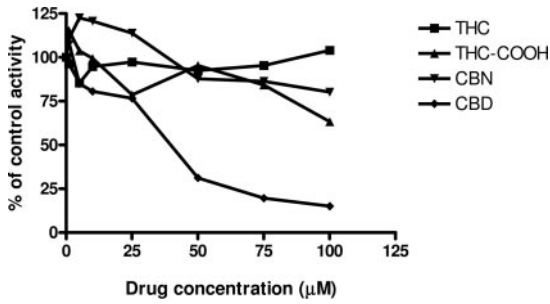


Fig. 2. Effects of THC, THC-COOH, CBN, and CBD on the VER-stimulated ATPase in P-gp expressed membranes. A fixed concentration of VER (10 µM) was incubated with different concentrations of cannabinoids. Values are the average of duplicate determinations.

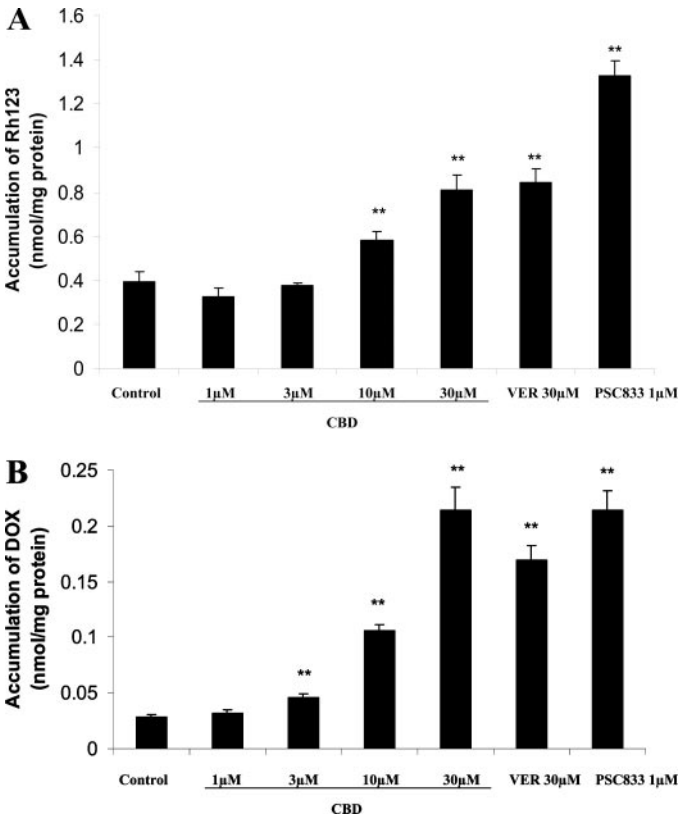


Fig. 3. Effect of CBD on the intracellular accumulation of Rh123 (A) and DOX (B) in Caco-2 cells. The intracellular accumulation of 5 µM Rh123 and 1 µM DOX in Caco-2 cells after 1-h incubation in the presence or absence of 1, 3, 10, and 30 µM CBD, 30 µM VER, or 1 µM PSC833 was determined as described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

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 was 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 and 100 µM CBD or 1 µM PSC833 (118.35 ± 3.33 , 216.90 ± 24.40 , and $258.69 \pm 26.27\%$ 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 $1166.59 \pm 71.18\%$ of the control by 5, 20, and 100 µM CBD and 1 µM PSC833, respectively ($p < 0.01$). As expected, in P-gp-nega-

tive LLC-PK1 cells, no significant effects on the accumulation of Rh123 or DOX were observed in the presence of either 100 µM CBD or 1 µM PSC833 (Fig. 4, A and B).

Flow-cytometry experiments also indicated that CBD increased the intracellular accumulation of Rh123 by inhibiting P-gp function in a concentration-dependent manner (Fig. 5). The IC_{50} 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 RBMEC 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 RBMEC 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 RBMEC monolayers (i.e., $P_{app\ B-A} \gg P_{app\ A-B}$). The $P_{app\ B-A}/P_{app\ A-B}$ ratio of Rh123 across Caco-2 and RBMEC 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 (Fig. 6). These data indicated that the treatments of 10 and 30 µM CBD resulted in 25.1 and 31.1% decreases of Rh123 $P_{app\ B-A}$ values and 75.6 and 116.7% increases of Rh123 $P_{app\ A-B}$ 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 (Fig. 7). After incubation for 120 min, a 52.5% decrease of $P_{app\ B-A}$ value and a 160.6% increase of

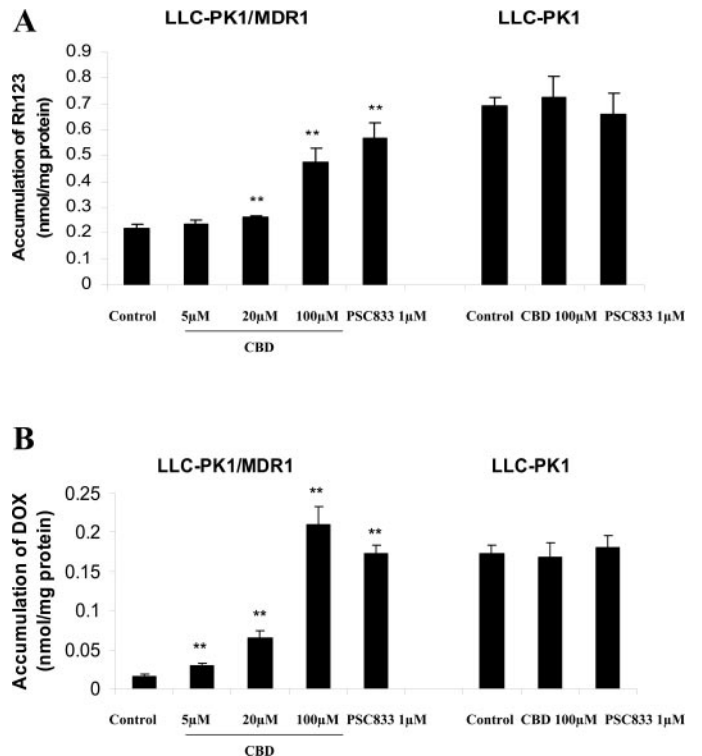


Fig. 4. Effect of CBD on the intracellular accumulation of Rh123 (A) and DOX (B) in LLC-PK1 and LLC-PK1/MDR1 cells. Inhibitory effect of CBD on P-gp function in LLC-PK1/MDR1 cells was assessed by measuring the intracellular concentrations of Rh123 and DOX after incubation for 1 h in the presence or absence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

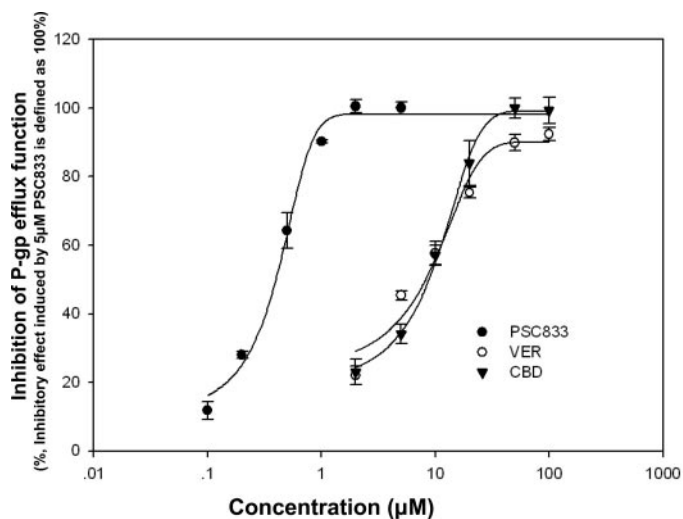


Fig. 5. Comparison of the concentration-dependent P-gp inhibitory effect of CBD, VER, and PSC833 by flow-cytometry assay. Flow cytometry was used to measure the accumulation of Rh123 in LLC-PK1/MDR1 cells after incubation with 2, 5, 10, 20, 50, and 100 μM CBD, 2, 5, 10, 20, 50, and 100 μM VER, or 0.1, 0.2, 0.5, 1, 2, and 5 μM PSC833 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 S.D., $n = 3$.

$P_{\text{app A-B}}$ value compared with the vehicle control were observed in the presence of 30 μM CBD (Table 2; $p < 0.01$).

Discussion

The P-gp-dependent ATPase assay is a widely used in vitro method to investigate the affinity of compounds for P-gp (Shapiro and Ling, 1994). It is unclear why some chemicals (VER, vinblastine, and DOX) stimulate ATPase activity, whereas others (cyclosporin A and 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 nonpsychoactive constituent CBD; and another major cannabinoid, CBN. The results indicated that all four compounds exhibited some degree of ATPase stimulation. Compared with our previous data testing various second-generation antipsychotic drugs (Boulton et al., 2002), THC-COOH ($V_{\text{max}}/K_m = 1.3$) and CBN ($V_{\text{max}}/K_m = 0.7$) have comparable ATPase activity with olanzapine ($V_{\text{max}}/K_m = 0.8$). Olanzapine was later confirmed by our laboratory to be a P-gp substrate in in vivo experiments using P-gp knockout 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 activ-

ity 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 less than that of VER, because the ATPase activity stimulated by 10 μM VER was not altered with coincubation of THC-COOH and CBN, even at a concentration of 100 μM . Interestingly, CBD had a potent inhibitory effect on the VER-stimulated ATPase activity, which occurred in a concentration-dependent manner. Therefore, further 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 (Figs. 3 and 4). The P-gp inhibitory potency of CBD ($\text{IC}_{50} = 8.44 \pm 0.58 \mu\text{M}$), determined by flow-cytometry assay in LLC-PK1/MDR1 cells, was much lower than that of PSC833 ($\text{IC}_{50} = 0.38 \pm 0.03 \mu\text{M}$) but comparable with that of another classic P-gp inhibitor, VER ($\text{IC}_{50} = 6.99 \pm 0.66 \mu\text{M}$). It is interesting that CBD seems to have a stronger effect on increasing the intracellular accumulation of DOX than that of Rh123 in both of the cell lines studied. The increase in the intracellular accumulation of Rh123 and DOX in Caco-2 cells produced by 30 μM CBD was 106.6 and 641.0% of control, respectively, whereas in LLC-PK1/MDR1 cells, 100 μM 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, 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). Organic cation transporter-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. Knockout 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,b). Coadministration 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 RBMEC 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 RBMEC monolayers

TABLE 2

Effect of CBD on P_{app} of 5 μM Rh123 across Caco-2 and RBMEC monolayers
Results are reported as mean \pm S.D., $n = 3$.

Cell	Inhibitor	P_{app}		$P_{app} \text{ B-A} / P_{app} \text{ A-B}$ Ratio
		B-A	A-B	
		$\text{cm s}^{-1} \times 10^7$		
Caco-2	Vehicle	26.30 \pm 1.41	0.78 \pm 0.05	33.72
	3 μM CBD	24.68 \pm 4.75	1.02 \pm 0.09*	24.20
	10 μM CBD	19.69 \pm 0.92**	1.37 \pm 0.38**	14.37
	30 μM CBD	18.13 \pm 1.99**	1.69 \pm 0.19**	10.73
	100 μM VER	4.92 \pm 0.57**	1.92 \pm 0.50**	2.56
RBMEC	Vehicle	11.98 \pm 0.30	0.99 \pm 0.09	12.10
	30 μM CBD	5.69 \pm 0.23**	2.58 \pm 0.19**	2.21
	100 μM VER	4.14 \pm 0.14**	3.24 \pm 0.10**	1.27

* $p < 0.05$, ** $p < 0.01$ versus vehicle control.

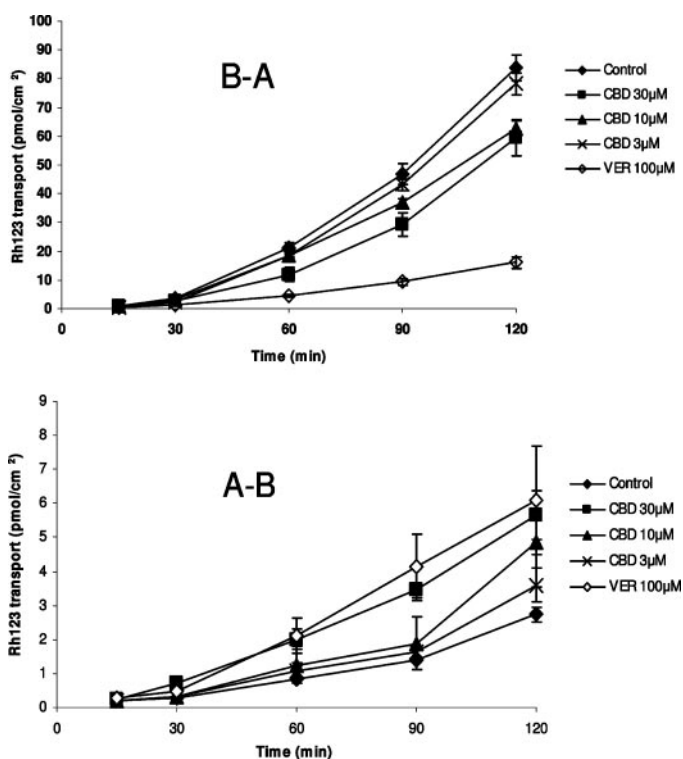


Fig. 6. Effect of CBD on the directional transport of Rh123 across Caco-2 cell monolayers. Rh123 (5 μM) was added to the basal (for B-A transport) or apical side (for A-B transport) of Caco-2 cell monolayers, whereas 3, 10, and 30 μM CBD or 100 μM VER were added to both sides of monolayers. The amounts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

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 RBMEC 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

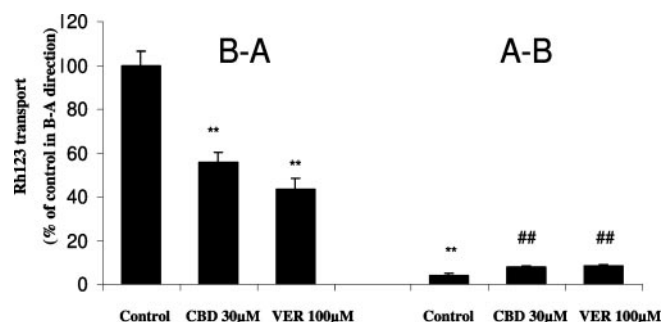


Fig. 7. Effect of CBD on the directional transport of Rh123 across the RBMEC monolayers. Rh123 (5 μM) was added to the donor compartment, whereas 30 μM CBD or 100 μM VER was added to both the apical and basal compartment. After 120-min incubation, concentration of Rh123 in receiver compartment was determined 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.

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 coadministered 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 that 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/day) for 6 weeks in patients with Huntington's disease (Consroe et al., 1991). A recent study indicated that the mean maximal concentration is approximately 3.0 nM obtained 60 min after oral administration of cannabis extract containing 5.4 mg of CBD (Nadulski et al., 2005). These concentrations are much lower than its in vitro effective concentration ($\sim 3 \mu\text{M}$). 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 administered 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 nonpsychoactive constituent in marijuana and exhibits many pharmacological properties, suggesting putative anticonvulsant, anxiolytic, antipsychotic, anti-nauseant, and anti-inflammatory effects (Mechoulam et al., 2002). Because of 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 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, and others, has 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, resulting in the inhibition of P-gp function.

In summary, a variety of in vitro methodologies and cell lines were used 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 coadministration 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 coadministered compounds that are P-gp substrates.

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