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Modulation of P-glycoprotein activity by cannabinoid molecules in HK-2 renal cells

*,[1]Paola Nieri, [2]Nadia Romiti, [1]Barbara Adinolfi, [1]Andrea Chicca, [3]Ilaria Massarelli & [2]Elisabetta Chieli

[1]Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa, Via Bonanno, 6, Pisa 56126, Pisa, Italia; [2]Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, Università di Pisa, Pisa, Italia and [3]Dipartimento di Chimica e Chimica Industriale, Università di Pisa, Pisa, Italia

1 Endogenous and synthetic cannabinoid molecules have been investigated as possible MDR-1/P-glycoprotein (P-gp) modulators in HK-2-immortalized renal cells, using calcein acetoxymethylester (calcein-AM) as a P-gp substrate.

2 Among the endocannabinoid molecules tested, anandamide (AEA), but not 2-arachidonoyl-glycerol (2-AG) or palmitoyl-ethanolamide (PEA), increased the intracellular fluorescence emitted by calcein, a metabolic derivative of the P-gp substrate calcein-AM, indicative of a reduction in transport capacity.

3 All the three synthetic cannabimimetics tested, that is, *R*-(+)-methanandamide (*R*(+)-MET), AM 251 and CP55,940 significantly increased calcein accumulation in the cytosol.

4 RT–PCR demonstrated that HK-2 cells do not express CB_1 or CB_2 cannabinoid receptors.

5 R(+)-MET, AM251 and CP55,940 were also evaluated as modulators of P-gp expression, by Western blot analysis. Only AM251 weakly enhanced the protein levels (by 1.2-fold) after a 4-day-long incubation with the noncytotoxic drug concentration 2 μM.

6 The present data provide the first evidence that the endocannabinoid AEA and different synthetic cannabinoids may inhibit the P-gp activity *in vitro via* a cannabinoid receptor-independent mechanism.
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Abbreviations: AEA, *N*-arachidonoyl-ethanolamide; 2-AG, arachidonoyl-glycerol; MDR, multidrug resistance; PEA, palmitoyl-ethanolamide; P-gp, P-glycoprotein; *R*(+)-MET, *R*(+)-methanandamide

Introduction

P-glycoprotein, (P-gp), encoded by the *MDR1* gene, is a membrane protein belonging to the ATP-binding cassette superfamily and it is a transmembrane efflux pump. Initially discovered in tumour cells while investigating the multidrug resistance mechanism, it has been also found and studied in many nontumour cells/tissues (Montano *et al*., 1996; Smit *et al*., 1998; Dautrey *et al*., 1999; Jonker *et al*., 1999; Gutmann *et al*., 2000; Florea *et al*., 2001; Batetta *et al*., 2003; Cisternino *et al*., 2003; Goralski *et al*., 2003; Elliott *et al*., 2004). P-gp indeed is involved in all pharmacokinetic events (Varma *et al*., 2003) and many single-nucleotide polymorphisms in the *MDR1* gene have been correlated with differences in individual drug responsiveness (Marzolini *et al*., 2004). P-gp shows a broad substrate specificity with a prevalent affinity for hydrophobic compounds; many natural and synthetic xenobiotics and endogenous molecules are P-gp substrates or modulators (Chan *et al*., 2004). *Cannabis sativa* derivatives are among the natural compounds influencing P-gp activity. In multidrug-resistant mouse lymphoma cells, cannabinol, cannabispirol and cannabidiol increase cytotoxic drug accumulation, whereas cannabidiolic acid, tetrahydrocannabidiolic acid and Δ^9-tetrahydrocannabinol (THC) reduce it (Molnar *et al*., 2000). In humans, many actions of THC are mimicked by endogenous molecules called endocannabinoids (De Petrocellis *et al*., 2004). *N*-arachidonoyl-ethanolamide (anandamide (AEA)) and 2-arachidonoyl-glycerol (2-AG) are the main endogenous agonists at cannabinoid receptors. Palmitoyl-ethanolamide (PEA) and other molecules are candidate members of the endocannabinoid mediator family (Facci *et al*., 1995; De Petrocellis *et al*., 2004; Bradshaw & Walker, 2005). Endocannabinoids are produced under physiological and pathological conditions by central and peripheral tissues, suggesting a possible therapeutic use of cannabimimetics or cannabinoid receptor antagonists in different pathologies (Goutopoulos & Makriyannis, 2002).

As no study has aimed to investigate the interaction between endocannabinoids or synthetic cannabinoids and P-gp until now, we tested the effect of some endogenous and synthetic cannabinoid molecules in regulating P-gp activity in HK-2 cells. This cell line retains many of the phenotypic and functional properties of *in vivo* proximal tubules (Ryan *et al*., 1994) and it is an useful *in vitro* model to study the *MDR1*-codified protein (Tramonti *et al*., 2001; Romiti *et al*., 2002; 2004).

*Author for correspondence; E-mail: paola.nieri@farm.unipi.it

Methods

Drugs

Verapamil (VP) and probenecid were obtained from Sigma Italia (Milan, Italy). AM251, *R*(+)-methanandamide (*R*(+)-MET), AEA, 2-AG, PEA and CP55,940 ((-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol) were purchased from Tocris Cookson Ltd (Avonmouth, U.K.). AEA and R(+)-MET were dissolved in ethanol, the other compounds in DMSO (stock solutions) and diluted in distilled water to the required concentrations on the day of experiment.

Cell culture

The immortalized proximal tubule epithelial cell line from normal adult human kidney (HK-2) was purchased from the American Type Cell Collection and cultured as described (Romiti *et al*., 2002). Briefly, cells were grown in DMEM/F12 medium supplemented with L-glutamine and antibiotics (penicillin and streptomycin), insulin, transferrin, Na-selenite, T3, hydrocortisone, prostaglandin E1 and 5% foetal calf serum. All chemicals, media and cell culture reagents were obtained from Sigma-Aldrich (Milan, Italy). Medium was changed three times a week and cells were subcultured weekly. All experiments were performed on confluent cells maintained for 48 h in serum-free medium. In experiments testing the effects of cannabinoids on P-gp expression, cells were cultured for 4 days in the presence of a single cannabinoid molecule or its vehicle before protein extraction. Preliminary tests were made to select cannabinoid concentrations that do not decrease cell viability.

Calcein-acetoxymethylester (calcein-AM) test

Evaluation of P-gp activity was performed by the fluorimetric measurement of the intracellular accumulation of calcein produced by ester hydrolysis of the P-gp substrate calcein-AM (Apotech, Geneva, Switzerland), as the transport capacity of P-gp is inversely proportional to the intracellular accumulation of fluorescent calcein (Hauser *et al*., 1998). Indeed, calcein is not a P-gp substrate and it cannot leave the cell *via* the plasma membrane, whereas the nonfluorescent calcein-AM is extruded from the MDR-1-expressing cells (Homolya *et al*., 1993). Calcein assay was performed in 96-well plates. HK-2 cells, cultured in microplates, were preincubated for 15 min with cannabinoid molecules. Thereafter, calcein-AM was added at 0.5 μM final concentration and calcein fluorescence measured after 1 h incubation at 37°C at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 538$ nm by a fluorescent plate reader (Fluoroskan II, Dasit, Italy). The acknowledged P-gp modulator VP was used as internal standard (positive control).

RNA isolation and reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was isolated from 10^6 cells using the 'SV total RNA isolation system kit', which includes a Dnase treatment (Promega, Italy). Reverse transcriptase reaction was performed with 'Qiagen OmniScript RT kit' (Qiagen, Milan, Italy) on 1.5 μg total RNA. Human CB_1 and CB_2 receptors were amplified using primers designed to optimize their specificity and thermodynamic properties by using the Oligo-Primer Analysis Software, v. 4.0. Primers were synthesized by Sigma-Genosys Ltd (Cambridge, U.K.). CB_1 forward (5′-CCACTCCCGCAGCCTCCG-3′) and CB_1 reverse (5′-ATGAGGCAAAACGCCACCAC-3′) primers yield a 294 bp product (517–810 position in Genbank accession no. X81120); CB_2 forward (5′-CGCCGGAAGCCCTCATAC-3′) and CB_2 reverse (5′-CCTCATTCGGGCCATTCTTG-3′) primers yield a 523 bp product (318–840 position in Genbank accession X74328). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as expression standard and amplified with forward (5′-GTGAAGGTCGGTGTCAACG-3′) and reverse (5′-GGTGAAGACGCCAGTAGACTC-3′) primers yielding a 300 bp product (85–384 position in Genbank accession NM_002046.2). PCR was performed with 'HotStartTaq Master Mix kit' (Qiagen, Milan, Italy) using 1/10 of cDNA in 12.5 μl final volume. PCR conditions were as follows: 95.0°C 15 min, followed by 1 min at 95.0°C, 1 min at 56.8°C, 1 min at 72.0°C (35 cycles) and then 10 min at 72.0°C for CB1; 95.0°C 15 min, followed by 1 min at 95.0°C, 1 min at 58.0°C, 1 min at 72.0°C (35 cycles) and then 10 min at 72.0°C for CB2; and 95.0°C 15 min, followed by 1 min at 95.0°C, 1 min at 55.0°C, 1 min at 72.0°C (36 cycles) and then 10 min at 72.0°C for GAPDH. All protocols were carried out in a MyCycler Thermalcycler (Bio-Rad, Milan, Italy) and PCR products were run on ethidium bromide-stained 1% agarose gel.

The DNA extracted from HK-2 cells (by using DNeasy Tissue kit (Qiagen, Milan, Italy)) was used as PCR-positive control, taking advantage from the fact that the amplified sequences belong to single exons.

PCR product identity was confirmed by sequencing (CRIBI Centre, University of Padova, Italy).

SDS–PAGE and Western blotting

P-gp was immunodetected by semiquantitative Western blot analysis. Crude membranes of HK-2 cells were obtained and extracted as described (Romiti *et al*., 2002). Ten micrograms of membrane proteins were separated by SDS–PAGE on 6% acrylamide Laemmli minigels and transferred overnight onto nitrocellulose membranes. Equal loading conditions in gels were routinely ascertained by staining blots with Ponceau-S. For immunoblotting, mdr Ab-1 polyclonal serum and peroxidase-conjugated secondary antibody (Inalco, Milan, Italy) were used. Blots were developed with the ECL detection system (Amersham Pharmacia Biotech, Italy) and analysed by densitometry (Gel Documentation System Chemi Doc and QuantiOne version 4.3 software, Bio-Rad, Milan, Italy).

Cytotoxicity assay

Cytotoxicity of cannabinoid compounds was tested to rule out the possibility that cytotoxic effects alter both the intracellular fluorescence in the calcein assay and the P-gp expression. Cells were grown in 96-multiwell plates in the absence or in the presence of cannabinoid molecules for 15 min to study effects of cannabinoids on P-gp activity or for 4 days to study effects of cannabinoids on P-gp expression. Cell viability was then evaluated by the WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene) colorimetric test (Roche,

Milan, Italy). WST-1 reagent undergoes intracellular cleavage by mitochondrial dehydrogenases in viable cells forming a dark red formazan product, which is quantified by absorbance at 450 nm. Briefly, WST-1 was added to each well and after 1 h incubation at 37°C, the absorbance was measured by a microplate reader (Wallac Victor II, Perkin-Elmer, MA, U.S.A.). In preliminary experiments, cytotoxicity of drug vehicles (never greater than 0.1% ($v\,v^{-1}$)) was excluded.

Computer-calculated logarithm of partition coefficient (ClogP) and solvent-accessible surface area (SASA)

Correlation between hydrophobicity and biological activity of cannabinoid molecules was tested as well. ClogP value is an accurate predictor of logP(ow) (log octanol/water partition coefficient) (Machatha & Yalkowsky, 2005) and it was calculated by means of the ChemDraw 6.0 program (CambridgeSoft, U.K.) by dissecting the solute under study into chemically meaningful fragments of known hydrophobicity. Molecular models were constructed by using standard geometries (standard bond lengths and angles) with the InsightII molecular modeling program (Biosym/MSI, San Diego, CA, U.S.A.) and a molecular mechanic approach was exploited for energy minimization of the molecules belonging to the entire data set, in order to obtain reasonable 3D arrangements. The cff91 force field was exploited as well. Energy minimizations were carried out with steepest descent and conjugate gradient minimization algorithms, till 0.001 kcal/mol Å root-mean-square deviation was achieved.

SASA and its polar and nonpolar fractions for each cannabinoids were calculated with the Homology module of InsightII.

Data evaluation and statistical analysis

The percent ratio between calcein-dependent fluorescence emission recorded from cells exposed to the drug and control cells was calculated to assess the P-gp activity. Similarly, in the cytotoxicity assay, the absorbance values are reported as % of control value (no treatment). Results are expressed as mean ± s.e.m. from n replicates. Differences among drug treatments were evaluated by the analysis of variance and the Bonferroni post-test. Immunoblotting results from treated cells were compared to immunoblotted protein in vehicle-treated cells after densitometric acquisition; differences between densitometric values for each treatment vs its control were evaluated by Student's t-test for unpaired data. $P \leqslant 0.05$ was taken to be significant.

Results

Functional studies

Among endocannabinoids, each tested at 20 μM and compared to VP at the same concentration, only AEA significantly enhanced the intracellular fluorescence after calcein-AM incubation (up to 159. 0 ± 10.8%, $n = 4$; $P \leqslant 0.05$, analysis of variance followed by Bonferroni post-test). In the presence of 2-AG or PEA, on the contrary, no significant difference with respect to control was evident (fluorescence with respect to control: 123.3 ± 16.9 and 101 ± 5.9%, respectively, $n = 4$ each) (Figure 1).

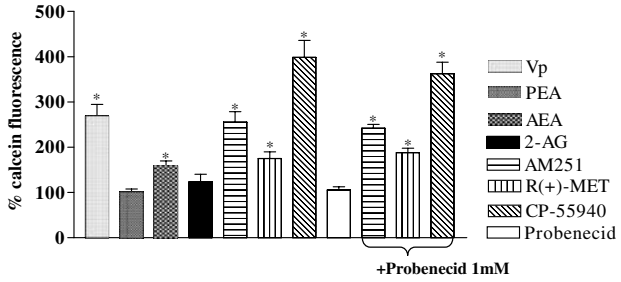

Figure 1 Effects of cannabinoid molecules and verapamil (standard compound) at 20 μM on calcein accumulation in HK-2 cells. Cells were preincubated for 15 min with the compounds and then calcein-AM 0.5 μM was added. After 1 h, intracellular dye concentration was evaluated by fluorimetry. The values are mean ± s.e.m. of four independent experiments. $P \leqslant 0.05$ using analysis of variance test followed by Bonferroni post-test was taken to be significant.

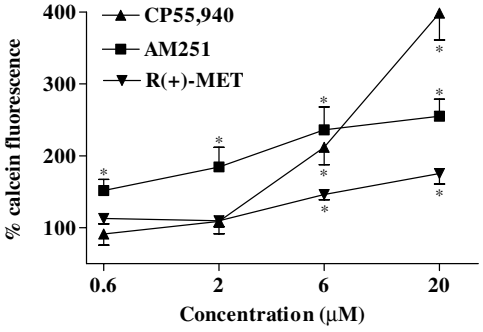

Figure 2 Concentration-dependent effects of AM251, CP55,940 and R(+)-MET on calcein accumulation in HK-2 cells. Cells were preincubated for 15 min with the tested compounds and then calcein-AM 0.5 μM was added. After 1 h, intracellular dye concentration was evaluated by fluorimetry. The values are mean ± s.e.m. of three independent experiments. $P \leqslant 0.05$ using analysis of variance test followed by Bonferroni post-test was taken to be significant.

Among synthetic cannabinoids, all molecules tested (20 μM each) significantly inhibited ($P \leqslant 0.05$) calcein-AM efflux from the cell. In the case of CP55,940 a particularly remarkable efflux inhibition appeared (398.7 ± 37.4% of control fluorescence, $n = 4$), which was higher than that of VP (269.2 ± 25.5%, $n = 4$). The inhibitor of multidrug resistance proteins (MRPs) probenecid (1 mM) did not significantly modify the basal calcein efflux in HK-2 cells and response to cannabinoids (Figure 1). This suggests that only P-gp modulation was responsible for the effects observed in calcein assay.

Figure 2 shows the concentration-dependent curves for the three synthetic cannabinoids $R(+)$-MET, CP55,940 and AM251 in the range 0.6–20 μM.

None of the tested compounds quenched the calcein fluorescence and no cytotoxicity was revealed after a time exposure correspondent to that used in calcein assay (data not shown).

P-gp expression by immunoblotting

Immunodetectable P-gp membrane content was not significantly changed after 4 days incubation with 0.6 and 2 μM

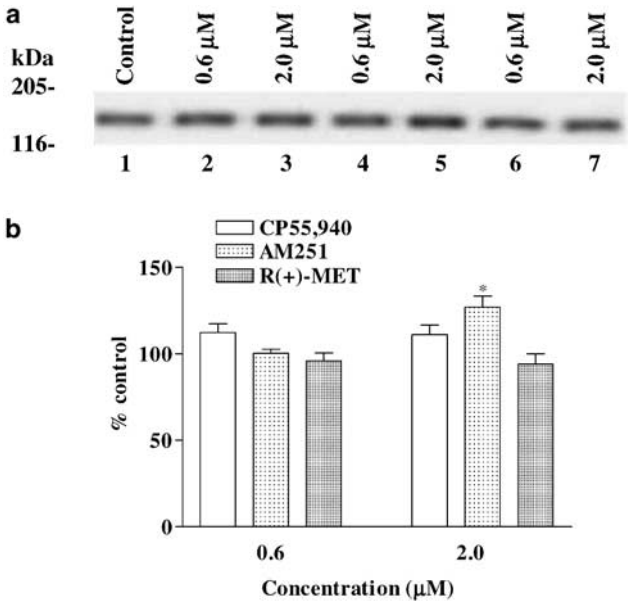

Figure 3 (a) Western blot analysis (representative photo of three experiments) for P-gp in crude membranes from HK-2 cells cultured for 4 days in basal serum-free medium (lane 1) or in serum-free medium supplemented with 0.6 and 2 μM CP55,940 (lanes 2,3), AM 251 (lanes 4,5) and R(+)-MET (lanes 6 and 7) and (b) Arbitrary densitometric values (mean $\pm$ s.e.m.) from the three experiments of Western blotting above described. One single control (C) representative of diluted DMSO or ethanol vehicle-treated cells is shown for simplicity as no influence was observed with respect to nontreated cells in any case. Statistical differences were determined by Student's *t*-test for unpaired data and $P \leqslant 0.05$ was taken to be significant.

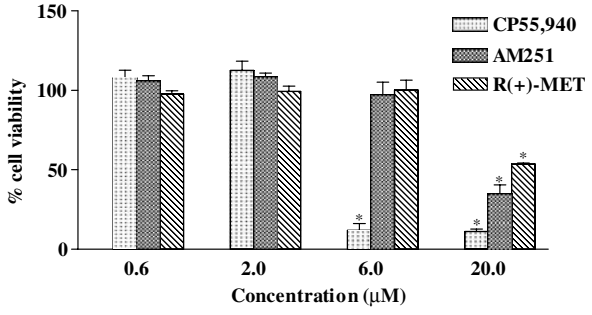

Figure 4 Cell viability of HK-2 cells as revealed by WST-1 colorimetric assay after 4 days incubation with the synthetic cannabinoids AM251, CP55,940 and R(+)-MET in the range 0.6–20 μM. Data are mean $\pm$ s.e.m. from four replicates. Significance of the difference of each point with the control value was obtained by analysis of variance and Bonferroni post-test; $P \leqslant 0.05$ was taken to be significant.

R(+)-MET or CP55,940. A modest but significant increase of the protein (by 20%, $n = 3$; $P \leqslant 0.05$; Student's *t*-test for unpaired data) was, on the other hand, observed in the presence of AM251 at 2 μM (Figure 3). At the concentrations 0.6 and 2 μM no cytotoxicity was observed by all the three cannabinoids, whereas a significant ($n = 3$; $P \leqslant 0.05$, analysis of variance followed by Bonferroni post-test) viability decrease was observed at 6 μM with CP55,940 and at 20 μM with all drugs (Figure 4).

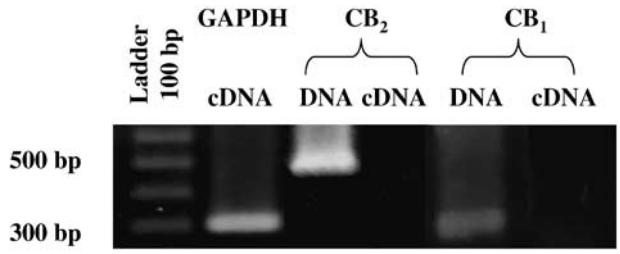

Figure 5 PCR products of CB_1 and CB_2 cannabinoid receptors from DNA of HK-2 cells and RT–PCR products of GAPDH (housekeeping gene), CB_1 and CB_2 receptors from total RNA of the same cells, as revealed after 1% agarose gel submarine electrophoresis with TBE 0.5 $\times$ buffer and EtBr staining. The photo is representative of three independent experiments. The size of PCR products was determined by comparison with 100 bp DNA ladder.

Table 1 ClogP, SASA and its polar and nonpolar fractions, for each cannabinoid tested as P-gp modulator

Compound	*ClogP*	*SASA*	*Polar*	*Nonpolar*
AEA	6.2	756.253	98.237	658.016
2-AG	6.9	793.086	111.031	682.055
PEA	6.0	673.908	98.162	575.75
R(+)-MET	6.5	784.208	73.01	711.196
AM251	7.0	710.727	51.904	445.784
CP55,940	5.8	718.374	133.343	585.031

CB1 and CB2 receptor expression by RT–PCR

Although DNA products of the expected size (294 bp for CB1 and 523 bp for CB2) and sequence were obtained by performing control PCRs on HK-2 cell DNA, and the control RT–PCR for GAPDH confirmed the quality of the cDNA template, by synthesizing a single 300 bp amplicon, no CB1 and CB2 receptor-specific RT–PCR products were obtained from total RNA of HK-2 cells (Figure 5).

ClogP and molecular surface areas

Table 1 shows the ClogP values of the tested compounds, ranging from 5.8 for CP55,940 to 7.0 for AM251. Values of SASA with the relative polar and nonpolar fractions are reported as well. No correlation between the P-gp-modulating activity of the cannabinoid compounds and their hydrophobicity or surface area-derived descriptors was found.

Discussion

Our results give the first evidence that an endocannabinoid molecule and some cannabinergics are modulators of the P-gp activity. Indeed, AEA was demonstrated to decrease calcein efflux *via* P-gp in cultured HK-2 renal cells. This activity was not observed with 2-AG and PEA. On the other hand, all the synthetic cannabinoids tested, that is, CP55,940, AM251 and *R*(+)-MET, had a similar effect to AEA, in the following order of P-gp inhibition potency: CP55,940 > AM251 > *R*(+)-MET.

The observed cannabinoid activity on P-gp does not seem to be CB receptor-mediated, because of the evidence that no mRNA encoding for CB_1 or CB_2 receptors can be detected with RT–PCR. On the other hand, the agonist/antagonist profile on CB receptors of the drugs under study already suggested the involvement of these receptors to be unlikely in the P-gp modulation. $R(+)$-MET and CP55,940 are, in fact, CB agonists, whereas AM251 has been extensively used as a CB_1 antagonist and more recently reported as CB_2 inverse agonist (New & Wong, 2003). In addition, the finding that the inhibitory potency of the stable AEA analogue was similar to that of AEA itself suggests that FAAH is also absent in HK-2 cells.

Although a functional response of canine tubular renal cells to the CB agonist CP55,940 has been reported, it was not blocked by CB antagonists, suggesting that the response was dissociated from the activation of CB receptors (Chou *et al*., 2001). Moreover, the renal expression of CB receptors and the AEA-degrading enzyme, FAAH, was found to be associated with the vasculature (Deutsch *et al*., 1997).

We found no evidence of cannabinoid cytotoxicity at the concentration and for the drug exposure time we adopted in the calcein assay. Cytotoxicity induced by P-gp inhibition in HK-2 cells has been reported by Zager (2001). In our experiments, a significant decrease in cell viability was observed only after a prolonged exposure (4 days) to cannabinoids and at concentrations higher than $2\,\mu$M. In addition, no correlation was observed at $6\,\mu$M CP55,940, AM251 or $R(+)$-MET between the drug inhibitory activity on P-gp and the extent of cytotoxicity 4 days later, although a positive correlation was observed when these cannabinoids were used at a higher concentration of $20\,\mu$M. Mechanisms underlying the different cytotoxic profile between drugs such as CP55,940 and AM251, which have at $6\,\mu$M similar inhibitory activities on P-gp efflux but different effects on cell survival, remain to be elucidated. Not even the influence on P-gp expression may give a rational explanation as only a modest increase (by 20%) of P-gp gene transcription by AM251 was observed. It seems therefore reasonable that other factors might be involved, for example, a different drug metabolism and/or cell responses other than those concurring with the P-gp inhibition.

The existence of a specific AEA transporter is actually debated (Glaser *et al*., 2003; Matthew *et al*., 2004) and MDR-1 protein has been postulated to be an AEA transmembrane transporter by Goutopoulos & Makriyannis (2002). In this study, we did not investigate the transmembrane transport of cannabinoids, but the P-gp inhibition we observed may be owing to competition of molecules with different hydrophobicity for the same transporter. Hydrophobic substances are typical substrates for P-gp (Zamora *et al*., 1988). Nevertheless, a correlation between steroid hydrophobicity and P-gp activity inhibition, reported by Yang *et al*. (1989), was not confirmed by other authors (Ueda *et al*., 1992; 1994; Hamilton *et al*., 2001). In addition, there is evidence that the P-gp hydrophobic substrates gain access to the transporter before leaving the inner side of the plasma membrane for the cytoplasm (Sharom, 2003) and a reduced polar surface area of a drug has been reported to correlate better than drug lipophilicity with an increased membrane permeation rate (Veber *et al*., 2002). Nevertheless, we did not observe any correlation between both the cannabinoid surface area descriptors, that is, SASA and its polar and nonpolar fractions and the cannabinoid ClogP values, and the inhibition of calcein efflux. This seems to suggest that the molecular mechanism of calcein efflux inhibition we observed is not based on the simple competition of molecules with different hydrophobicity for the same transporter, such as a cannabinoid and calcein-AM. In this regard, Seelig & Landwojtowicz (2000) observed that although the partitioning into the membrane was the rate-limiting step for P-gp-substrate interaction, dissociation of the P-gp–substrate complex depended on the number and strength of the hydrogen bonds between the substrate and the membrane embedded region of the transporter. Furthermore, specific structural elements that are critical for the recognition by P-gp (Pearce *et al*., 1989) and different drug binding sites on the protein (Martin *et al*., 2000) has been suggested.

Irrespective to the molecular mechanism of action, the effect of AEA on P-gp activity lead us to hypothesize a role for the endocannabinoid system in regulating the P-gp activity in kidney and other districts such as intestine, liver and blood-brain barrier where P-gp is expressed and involved in the physiological processes of absorption, distribution, excretion and metabolism (Varma *et al*., 2003). Similarly, synthetic cannabinoids involved in therapeutic regimens (Tanigawara, 2000) may have a role in modifying the pharmacokinetic profile and the bioavailability of other drugs. In this perspective, it seems noteworthy to us that a drug structurally similar to AM251, SR141716A (rimonabant®), is currently undergoing clinical trials for obesity, smoking cessation and alcohol abuse (Fernandez & Allison, 2004).

In conclusion, our data show that P-gp activity may be modulated by the endocannabinoid system and by synthetic cannabinoids in HK-2 cells, in a CB1- and CB2-receptor-independent pathway. This opens an interesting scenario in kidney physiology and in the pharmacology and therapeutic potential of cannabinoid drugs.

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