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The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells

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MDR, multiple drug resistance
P-gp, P-glycoprotein
THC, Δ^9-tetrahydrocannabinol
CBD, cannabidiol
CBN, cannabinol
Rh123, Rhodamine 123
WIN, WIN 55, 212-2
Et-743, Ecteinascidin-743

ABSTRACT

Cannabis is the most widely used illicit drug in the world. Cannabinoids are used therapeutically by some patients as they have analgesic, anti-emetic and appetite stimulant properties which palliate adverse symptoms. Use of these agents in an oncology setting raises the question of whether they act to modulate the effectiveness of concurrently administered anti-cancer drugs. The transporter, P-glycoprotein (P-gp) confers multiple drug resistance (MDR) by effluxing a diverse array of anti-cancer agents. This study was undertaken to examine the effect of cannabinoids on P-gp. Unlike the known P-gp inhibitor, PSC833, short 1 h exposure to three plant-derived cannabinoids, cannabinol (CBN), cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) and the synthetic cannabinoid receptor agonist, WIN55, 212-2 (WIN) did not inhibit the efflux of the P-gp substrate Rhodamine 123 (Rh123) in either a drug-selected human T lymphoblastoid leukaemia cell line (CEM/VLB_{100}) or in a mouse fibroblast *MDR1* transfected cell line (77.1). However, in CEM/VLB_{100} cells, prolonged 72 h exposure to the cannabinoids, THC and CBD, decreased P-gp expression to a similar extent as the flavonoid, curcumin (turmeric). This correlated with an increase in intracellular accumulation of Rh123 and enhanced sensitivity of the cells to the cytotoxic actions of the P-gp substrate, vinblastine. Taken together, these results provide preliminary evidence that cannabinoids do not exacerbate P-gp mediated MDR. Further, plant-derived cannabinoids are moderately effective in reversing MDR in CEM/VLB_{100} cells by decreasing P-gp expression.

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1. Introduction

Cannabinoids are plant-derived, synthetic or endogenous compounds that produce their pharmacological actions by interacting with the endocannabinoid system [1]. In recent times there has been a resurgence of interest in the therapeutic application of cannabinoids with many governments decriminalizing the use of these compounds for medicinal purposes. Indeed, clinical studies have provided evidence for the effectiveness of cannabinoids in treating conditions such as multiple sclerosis [2] and chronic neuropathic pain [3]. In the treatment of cancer, the analgesic, anti-emetic and mood enhancing qualities of cannabinoids have justified their use as palliative care agents [4]. The emerging use of cannabinoids in this context raises the important question of whether they act to modulate the effectiveness of concurrently administered anti-cancer treatments.

One of the major issues influencing the outcome of anticancer treatment is the ability of tumour cells to develop resistance to chemotherapeutic agents [5]. Of particular concern is the phenomenon of multiple drug resistance (MDR). This form of resistance results from a mechanism that simultaneously confers resistance to many xenobiotics despite an unrelated chemical structure or target of action [6]. The most extensively characterised mechanism of MDR results from the over-expression of the ATP-binding cassette transporter, P-glycoprotein (P-gp). P-gp acts in an energy dependent manner to efflux a diverse range of large, hydrophobic, clinically employed anticancer agents such as anthracyclines, vinca alkaloids, taxol and podophyllotoxin derivatives [7]. Efflux prevents intracellular accumulation of the anticancer agents and subsequent interaction with their respective drug targets.

Clinical studies have demonstrated that human tumours arising from tissues which constitutively express P-gp are inherently multidrug resistant [8]. P-gp expression is also found de novo in a variety of lymphomas and leukaemias, or on relapse following chemotherapy induction [9]. The expression of P-gp is considered an adverse prognostic factor in adult acute myeloid leukaemia as it is strongly correlated with a reduced remission rate and a higher incidence of refractory disease [10,11]. Given the clinical relevance of P-gp mediated MDR, considerable effort has been expended in developing compounds capable of modulating P-gp activity as a means of reversing the MDR phenotype. Such compounds have two potential mechanisms through which they may act to increase the accumulation and efficacy of chemotherapeutic agents: (1) functional inhibition of P-gp mediated transport and/or (2) a reduction in P-gp expression.

There have been many functional inhibitors of P-gp identified, including verapamil [12] and PSC833 [13]. However, the results of clinical trials with these agents were disappointing due to dose limiting toxic effects of the modulator [14] or increased toxicity of the co-administered chemotherapies [15]. Considerably fewer compounds that down-regulate P-gp expression have been identified. Two such compounds are curcumin [16,17] and Ecteinascidin-743 (Et-743) [18]. However, neither of these compounds are yet to reach the clinical trial stage of development for their MDR modulating properties. Accordingly, the search for clinically applicable P-gp modulators continues.

Over 60 different cannabinoid compounds are produced by the cannabis plant, *Cannabis sativa*. Three well-characterized plant-derived cannabinoids are the main psychoactive constituent, Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN). It has been previously reported that CBN (6.44 μM) and CBD (6.34 μM) functionally inhibit P-gp mediated efflux of the fluorescent substrate Rhodamine 123 (Rh123) in a *MDR1* transfected mouse T lymphoma cell line (L5178) [19]. Given the need to find a clinically applicable MDR reversal agent this preliminary finding warrants further investigation. Furthermore, the increasing use of cannabinoids in the palliative care of cancer patients reinforces the need for studies assessing the interaction of these drugs with proteins responsible for MDR, such as P-gp.

The present study was undertaken to further characterize the ability of cannabinoids to act as P-gp mediated MDR reversal agents. Three plant-derived cannabinoids, CBN, CBD and THC and the synthetic cannabinoid, WIN55, 212-2 (WIN) were assayed for their ability to act as functional inhibitors of P-gp. In addition, the possibility that CBD and THC may down-regulate P-gp expression in a drug selected human T lymphoblastoid cell line was investigated.

2. Materials and methods

2.1. Cell lines and culture conditions

The acute T lymphoblastoid leukaemia cell line (CCRF-CEM) and the P-gp over expressing, multidrug resistant sub line (CEM/VLB_{100}), were kindly donated by Prof. M. Haber (Children's Cancer Institute Australia, Sydney, NSW, Australia). The CEM/VLB_{100} cell line expresses high levels of P-gp as a result of *MDR1* gene amplification [20,21]. Both cell lines were cultured in RPMI-1640 (Invitrogen Australia, Mount Waverly, VIC) supplemented with 10% (v/v) foetal calf serum (FCS) (Invitrogen Australia, Mount Waverly, VIC). Cell density was maintained between 10^5 and 10^6 cells/ml, and cultures were limited to 12 consecutive passages. Mouse 77.1 fibroblast cells lacking functional *Mdr1a* and *Mdr1b* genes and transfected with human *MDR1* cDNA [22] were kindly provided by Prof. A. Schinkel (Netherlands Cancer Institute, Amsterdam). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Australia, Mount Waverly, VIC) supplemented with 10% (v/v) FCS. Cell cultures were maintained within the exponential phase of growth. Cell cultures were maintained within the exponential phase of growth in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

2.2. Cell viability assays

The MTS assay is a colorimetric assay for mitochondrial oxidative metabolism that is used as a measure of cell viability. In order to determine the maximum sub-lethal concentrations of CBN, CBD (Australian Government Analytical Laboratories, Pymble, NSW), THC (Sigma–Aldrich, Sydney, NSW) and WIN (kindly donated by A/Prof. I. McGregor, Department of Psychology, University of Sydney, NSW,

Australia), CCRF-CEM or CEM/VLB_{100} cells were plated at 5×10^4 cells/well in 96-well plates under the culture conditions described above and exposed to increasing doses of the cannabinoids for 72 h. The ethanol concentration was 0.1% (v/v) for all treatments. CellTiter 96® AQ_{ueous} MTS Reagent (Promega Corporation, Annandale, NSW) mixed 20:1 with phenazine methosulfate (Sigma–Aldrich, Sydney, NSW) was added to a final concentration of 10% (v/v). The plates were incubated for 4 h at 37 °C and the production of the formazan product monitored by measuring the A_{490} on a Bio-rad 3550 Microplate Reader. The concentration required to inhibit cell growth by 50% (IC_{50}) was calculated using GraphPad® Prism.

The MTS assay was also used to assess whether cannabinoids alter the potency of the antiproliferative effects of vinblastine. CCRF-CEM or CEM/VLB_{100} cells were exposed to 0.1 nM to 10 μM vinblastine sulfate (Sigma–Aldrich, Sydney, NSW) for 72 h, either alone, or in the presence of sub-lethal concentrations of verapamil hydrochloride, CBD or THC (10 μM). The dose of verapamil employed in this study has previously been shown to be sub-toxic in both the CCRF-CEM and CEM/VLB_{100} cell lines [23].

2.3. Rhodamine accumulation assays

CCRF-CEM or CEM/VLB_{100} cells (5×10^5/sample) were incubated with vehicle, PSC833 (1 μM) (Novartis, Sydney, NSW), CBN, CBD, THC (0.1–10 μM), or WIN (0.03–3 μM) for 10 min prior to the addition of Rh123 (1 μM) (Invitrogen Australia, Mount Waverly, VIC) and then further incubated for 60 min in the dark, at 37 °C. A final concentration of 0.13% (v/v) ethanol was used for all experiments and controls. Cells were then transferred to ice and maintained at 0 °C for all subsequent steps. The cells were washed in PBS containing FCS (1% v/v), and propidium iodide (0.5 μg/ml) (Sigma–Aldrich, Sydney NSW) was added. Cells were analysed on a FACScan flow cytometer with a 488 nm argon laser using CellQuest™ software (BD, Sydney, NSW). Rh123 fluorescence was measured by a 530 nm band-pass filter and propidium iodide fluorescence measured with a 585 nm band-pass filter. Gates were set to exclude propidium iodide positive cells and clumps and debris on the basis of forward and side scatter.

Mouse 77.1 fibroblasts were plated at 5×10^4 cells/well into 24-well plates and allowed to attach overnight. The medium was then removed and replaced with medium containing either vehicle, PSC833 (1 μM), CBN (10 μM), CBD (10 μM) THC (10 μM) or WIN (3 μM) and allowed to incubate for 10 min at 37 °C before the addition of Rh123 (1 μM). A final concentration of 0.15% (v/v) ethanol was used for all experiments and controls. Cells were incubated in the dark for 60 min at 37 °C, then transferred to ice and maintained at 0 °C whilst they were harvested by treatment with trypsin–EDTA for 15 min, dislodged by pipetting, washed and analysed as described above.

CEM/VLB_{100} cells at 9×10^4 cells/ml were treated with vehicle, verapamil (10 μM), curcumin (10 μM) (Sigma–Aldrich, Sydney, NSW), CBD or THC (1–10 μM) for 72 h. Cells were then harvested by centrifugation and washed several times in PBS to ensure complete removal of the compounds. Cells were incubated with Rh123 (1 μM) in the dark for 60 min, 37 °C, washed and analysed as described above.

2.4. Protein extraction and Western blot analysis

Crude membrane preparations were extracted from CEM/VLB_{100} cells treated with vehicle, curcumin (10 μM), CBD or THC (0.1–1 μM) for 72 h by resuspending the cells in homogenisation buffer (30 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl flouride containing mini-protease inhibitor cocktail (Roche Diagnostics, Castle Hill, NSW)) and incubating on ice for 45 min. Cells were then centrifuged at 19 000 rpm for 30 min at 4 °C and the resulting pellet resuspended in homogenisation buffer containing Triton X-100 (1% v/v) and incubated at 4 °C for 1 h before being centrifuged again at 19 000 rpm for 30 min at 4 °C. The supernatant containing the membrane fraction was collected and analysed for its protein concentration using the Pierce BCA Protein Assay Kit (Progen Biosciences, Archerfield, QLD).

Whole cell lysates were prepared from CEM/VLB_{100} cells treated for 72 h with vehicle, curcumin (10 μM), CBD or THC (0.1–10 μM) by resuspending the cells in lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1% (v/v) Triton X-100 containing mini-protease inhibitor cocktail). Several cycles of freeze–thaw were undertaken before nuclei were pelleted by centrifugation at 14 000 rpm for 5 min at 4 °C and the supernatant containing cellular proteins collected. Protein concentration was determined using the Pierce BCA Protein Assay Kit.

Cell membrane (2.5 μg/lane) or whole cell lysate (20 μg/lane) proteins were separated on a SDS-polyacrylamide gel (10% v/v) and immunoblotted onto a PVDF membrane (Bio-Rad Laboratories, Regents Park, NSW). The membranes were blocked overnight in 10% skim milk powder. For P-gp detection, the membranes were incubated sequentially with Zymed Mouse anti-Multidrug Resistance 1 + 3, clone C219 (BioScientific Pty Ltd, Gymea, NSW) and Anti-Mouse IgG (H + L), HRP Conjugate (Promega Corporation, Annandale, NSW). Alpha tubulin was detected by sequential incubation with Abcam rat monoclonal YL1/2 anti-alpha tubulin antibody (Sapphire Biosciences Pty Ltd, Redfern, NSW) and Anti-Rat IgG (H + L), HRP conjugate (Santa Cruz Biotechnology Inc, Santa Cruz, California). Proteins were detected with Pierce Super-Signal West Dura Extended Duration Substrate (Progen Biosciences, Archerfield, QLD) and the membranes visualised using a Kodak™ Image Station 440CF (Perkin-Elmer Life Sciences, Melbourne, VIC). Densiometry was performed using KODAK 1D Image Analysis Software, version 3.6. The densiometric values for P-gp in whole cell lysates were normalised to the respective value for alpha tubulin to ensure that any variation in loading between samples was compensated for. Equal loading and transfer of protein was confirmed for the membrane extracts using SYPRO® Ruby protein blot stain (Invitrogen Australia, Mount Waverly, VIC), as the extraction method employed precluded the use of a cytoplasmic housekeeping protein such as alpha tubulin.

2.5. Statistical analysis

Data are expressed as the mean + S.E.M. from at least three independent experiments. Differences between the means were analysed using an unpaired Student's *t*-test. Two-factor analysis of variance (ANOVA) was also performed to examine

Table 1 – The highest non-toxic dose and IC_{50} for cannabinoid compounds in CCRF-CEM and CEM/VLB_{100} cells

Compound	IC_{50} ± S.E.M.	Highest non-lethal dose (μM)
CCRF-CEM		
CBN (μM)	36 ± 0.9	24
CBD (μM)	22 ± 0.6	13
THC (μM)	38 ± 1.3	24
WIN (μM)	13.5 ± 0.2	3.5
Vinblastine (nM)	4.8 ± 0.9	ND
Colchicine (nM)	7.8 ± 0.5	ND
CEM/VLB_{100}		
CBN (μM)	38 ± 1.0	24
CBD (μM)	20 ± 0.7	13
THC (μM)	39 ± 1.7	24
WIN (μM)	16 ± 0.6	4
Vinblastine (nM)	610 ± 60.5	ND
Colchicine (nM)	438 ± 64.1	ND

Data is derived from three independent experiments, each performed in quadruplicate and expressed as the mean ± S.E.M. IC_{50}. Vinblastine and colchicine are known P-gp substrates. ND: not determined.

any differences in the effects of the cannabinoids on P-gp expression in crude membrane versus whole cell lysate extracts. Results are denoted according to significance; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. *Determining the highest sublethal dose*

The MTS assay of cell viability was employed to determine the maximum sub-toxic dose of the cannabinoid compounds. Table 1 shows the maximum concentration of each compound for which 100% cell viability was maintained. There was no significant difference in the IC_{50} between the CCRF-CEM and CEM/VLB_{100} cell lines for any of the cannabinoid compounds assayed. There was, however, a significant increase in the IC_{50} for the known P-gp substrates, vinblastine and colchicine in the CEM/VLB_{100} compared to CCRF-CEM cells, consistent with these drugs being subject to P-gp mediated efflux.

3.2. *Cannabinoid effects on P-gp efflux activity*

In order to examine the effect of cannabinoids on the efflux activity of P-gp the intracellular accumulation of Rh123, a fluorescent P-gp substrate, was assayed by flow cytometry. As can be seen in Fig. 1, Rh123 accumulation in the high P-gp expressing, CEM/VLB_{100} cells was significantly reduced compared to the parental CCRF-CEM cell line. This is consistent with the relative level of P-gp expression observed by Western blot where the CEM/VLB_{100} cells express high levels of P-gp compared to the non-drug selected CCRF-CEM cell line in which P-gp was not detected (data not shown). The potent P-gp inhibitor, PSC833, restored Rh123 accumulation in the CEM/VLB_{100} cells to that of the CCRF-CEM cell line. However, exposure to sub-lethal concentrations of the cannabinoids,

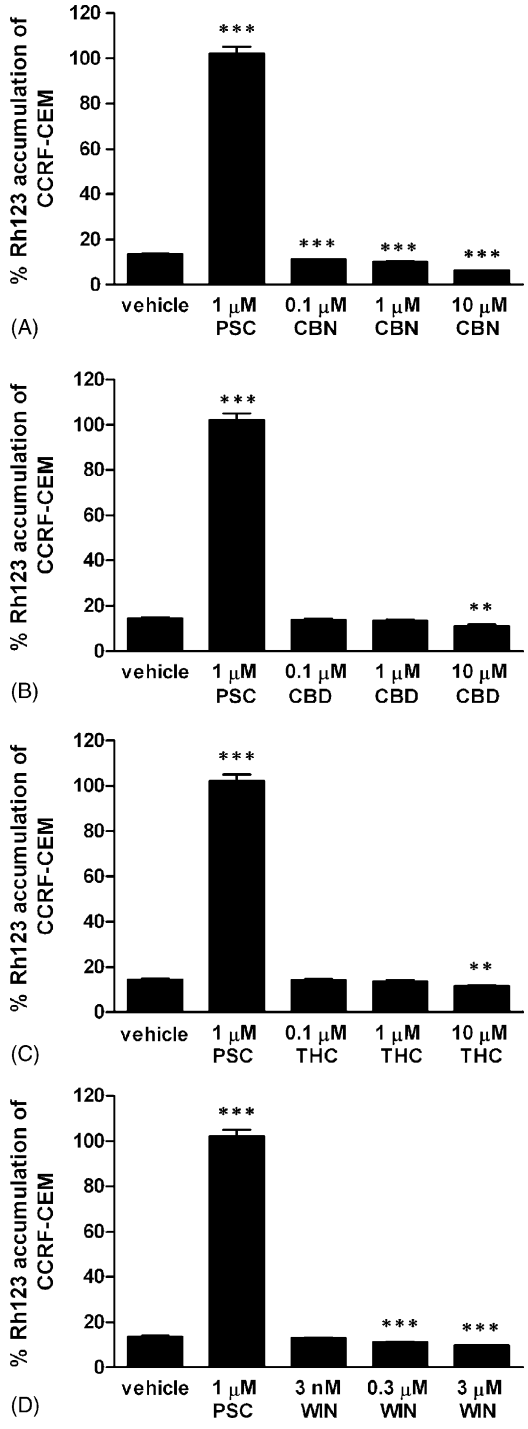

Fig. 1 – The effect of cannabinoids on the accumulation of Rh123 in CEM/VLB_{100} cells. The data for each cannabinoid: (A) CBN, (B) CBD, (C) THC, (D) WIN is derived from three independent experiments performed in quadruplicate and expressed as the mean percentage Rh123 accumulation + S.E.M. of the vehicle treated, drug sensitive CCRF-CEM cell line. PSC833 (1 μM PSC) was employed as a positive control for P-gp efflux inhibition. d.f. = 4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

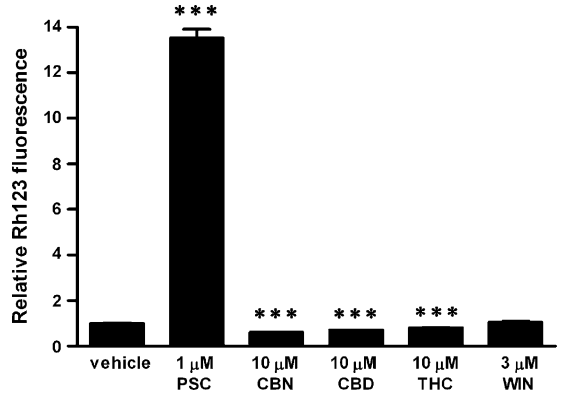

Fig. 2 – The effect of cannabinoids on Rh123 accumulation in *MDR1* transfected 77.1 cells. The data is derived from three independent experiments performed in quadruplicate and expressed as the mean relative Rh123 accumulation + S.E.M. of vehicle treated cells. PSC833 (1 μM PSC) was employed as a positive control for P-gp efflux inhibition. d.f. = 6, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CBN, CBD, THC and WIN for 1 h did not reduce the Rh123 accumulation deficit observed in the CEM/VLB_{100} cell line. Rather, the cannabinoids promoted a small but significant decrease in Rh123 accumulation (Fig. 1A–D). This decrease in Rh123 accumulation is unlikely to be mediated by cannabinoid enhancement of P-gp substrate transport as the cannabinoids also produced a similar effect on Rh123 accumulation in the CCRF-CEM cells (data not shown).

It has been reported that similar concentrations of CBN and CBD reversed P-gp mediated efflux of Rh123 in a *MDR1* transfected cell line [19]. As our findings conflict with this previous report, we assayed the effect of cannabinoids on Rh123 accumulation in *MDR1* transfected mouse 77.1 fibroblast cells that lack functional *Mdr1a* and *Mdr1b* genes [22]. As shown in Fig. 2, the P-gp inhibitor, PSC833, markedly increased Rh123 accumulation compared to the vehicle treated cells. However, none of the cannabinoids increased the accumulation of Rh123 relative to vehicle.

3.3. *The effect of cannabinoids on vinblastine cytotoxicity*

As short incubation periods with cannabinoids were ineffective in modulating P-gp, we then decided to examine the effects of longer incubation times on P-gp activity. We did this first by observing the effect of concurrent cannabinoid exposure on the cytotoxicity of the P-gp substrate vinblastine. In these studies we chose to focus on THC and CBD as these compounds are those found abundantly in the plant and also in new formulations being increasingly used by patients such as Sativex® [24,25]. Table 2 displays the IC_{50} of vinblastine and the fold reversal of resistance compared to the vehicle treated controls. In the CEM/VLB_{100} cell line, verapamil was the most effective at reversing P-gp mediated resistance, producing a 26.4 fold reduction in the IC_{50} of vinblastine. At a concentration of 10 μM both CBD and THC reduced the IC_{50} of vinblastine by approximately three-fold. This effect was unique to the CEM/VLB_{100} cell line, as neither of the cannabinoids nor verapamil significantly affected the IC_{50} of vinblastine in the CCRF-CEM cell line. This finding suggests that the reversal of resistance to vinblastine upon exposure to these cannabinoids is P-gp specific.

Table 2 – The effect of cannabinoids on vinblastine cytotoxicity in CCRF-CEM and CEM/VLB_{100} cells

Modulator	IC_{50} (nM)	Fold reversal	Statistical significance
CCRF-CEM			
Vehicle	4.8 ± 0.5		–
10 μM verapamil	3.1 ± 0.4	1.5	NS
10 μM CBD	4.4 ± 0.8	1.1	NS
10 μM THC	4.7 ± 0.6	1.0	NS
CEM/VLB_{100}			
Vehicle	609.8 ± 34.9		–
10 μM verapamil	23.1 ± 8.0	26.4	***
10 μM CBD	188.9 ± 34.9	3.2	***
10 μM THC	218.5 ± 43.1	2.8	***

Data is derived from three independent experiments performed in quadruplicate and expressed as the mean ± S.E.M. IC_{50} for vinblastine. Verapamil is a P-gp inhibitor that was employed as a positive control. Results are expressed relative to the vehicle treated group for the corresponding cell line. NS: not significantly different to vehicle. d.f. = 4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.4. *Seventy-two-hour cannabinoid exposure reduces P-gp expression levels*

Since 1 h exposure to cannabinoids did not affect Rh123 accumulation, the observed increase in vinblastine potency after 72 h co-incubation with the cannabinoids is unlikely to be mediated by direct inhibition of P-gp substrate transport. In order to test whether the increase in vinblastine potency after 72 h co-incubation with cannabinoids is due to a reduction in P-gp expression, we employed two methods.

The first measured the functional consequences of long-term cannabinoid pre-exposure on P-gp activity without cannabinoids being present at the time of testing. In order to do this, CEM/VLB_{100} cells were incubated with cannabinoids for 72 h then thoroughly washed, before the accumulation of Rh123 was assessed in the absence of cannabinoids. Accordingly, any effects observed on Rh123 accumulation are likely to result from the effects of cannabinoid pre-exposure on the amount of P-gp present in the cell membrane, as they cannot be attributed to any direct cannabinoid modulation of P-gp transport. Verapamil, an inhibitor of P-gp efflux activity was employed as a control to demonstrate that all compounds had been completely removed and that any observed effects were not due to direct inhibition of P-gp efflux activity. Curcumin is a compound found in turmeric that down-regulates P-gp expression after 72 h exposure [16]. It was used in the present context to demonstrate that down-regulation of P-gp expression would increase Rh123 accumulation in the CEM/VLB_{100} cells. As shown in Fig. 3, verapamil did not significantly affect Rh123 accumulation indicating that all compounds had been adequately removed from the cells prior to exposure to the substrate. The cannabinoids, CBD

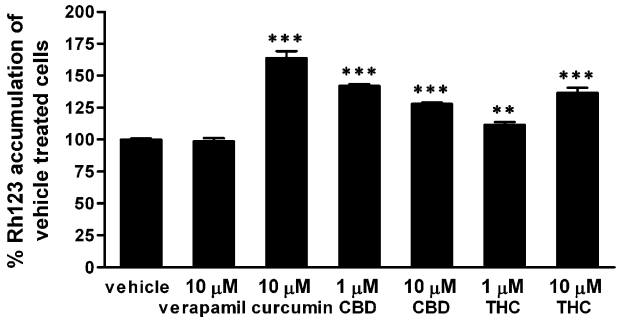

Fig. 3 – The effect of pre-incubation with cannabinoids for 72 h on Rh123 accumulation in CEM/VLB$_{100}$ cells. The data is representative of the mean percentage + S.E.M. Rh123 accumulation of vehicle treated cells from three independent experiments, each performed in quadruplicate. Verapamil was employed as a control to demonstrate that effects are not the result of functional inhibition. Curcumin was employed as a control for down-regulation of P-gp expression. d.f. = 4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and THC increased Rh123 accumulation, albeit, to a lesser extent than curcumin.

The second method we used to investigate whether long-term cannabinoid exposure decreases P-gp expression is Western blotting. CEM/VLB$_{100}$ cells were exposed to the cannabinoids for 72 h and the level of P-gp present in the cell membrane or the whole cell extract was assessed. As seen in Fig. 4, 10 μM curcumin treatment significantly reduced P-gp expression by approximately 50% in the membrane extracts of CEM/VLB$_{100}$ cells. Both CBD and THC (1–10 μM) also significantly reduced the amount of P-gp present in the membrane fraction to a level comparable to that observed for curcumin. Surprisingly, curcumin did not reduce the amount of P-gp in whole cell lysates of CEM/VLB$_{100}$ cells. However, the cannabinoids THC and CBD were effective at reducing P-gp levels from whole cell lysates, even at low 0.1 μM concentrations. Two factor ANOVA confirmed that both the cannabinoids, THC ($F_{1,3} = 5.3$, $p < 0.05$) and CBD ($F_{1,3} = 5.1$, $p < 0.05$), were overall more effective in reducing P-gp expression in whole cell lysates than in crude membrane extracts.

These Western blotting results correlate well with our functional assay of altered P-gp expression following THC and CBD exposure. Taken together, the results of these two independent assays provide strong evidence that 72 h cannabinoid exposure reduces the expression of P-gp in CEM/VLB$_{100}$ cells.

4. Discussion

This study examined the effect of cannabinoid compounds on P-gp activity and expression in MDR cell lines. It was found that 1 h cannabinoid exposure does not inhibit P-gp activity in drug selected human MDR leukaemia cells (CEM/VLB$_{100}$) or *MDR1*-transfected, mouse fibroblast cells (77.1). However, prolonged 72 h exposure to the cannabinoids, THC and CBD decreased P-gp expression in the CEM/VLB$_{100}$ cells, with a concomitant increase in intracellular Rh123 accumulation and enhanced sensitivity to the cytotoxic actions of the P-gp substrate, vinblastine.

Accumulating evidence documents the antiproliferative actions of cannabinoids on a diverse array of tumorigenic cell lines [26–28], including those of haematopoietic origin [29–31]. The current study shows that CBN, CBD, THC and WIN reduced the cell viability of a human T lymphoblastoid leukaemia (CCRF-CEM) cell line in the low micromolar concentration range (see Table 1). This is consistent with the finding that THC induces apoptosis in a CB_1/CB_2 receptor independent manner in a panel of leukaemia cell lines,

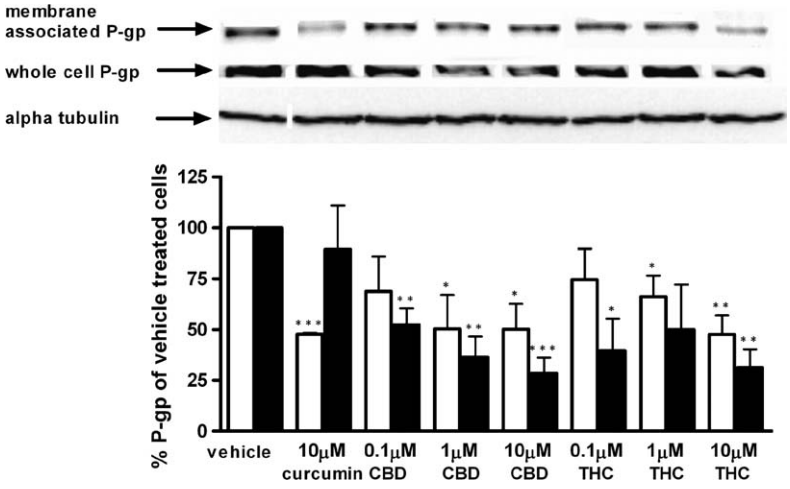

Fig. 4 – The effect of 72 h incubation with CBD and THC on the amount of P-gp present in CEM/VLB$_{100}$ cells. Data is derived from three independent experiments for each extraction method employed and expressed as the mean + S.E.M. percentage P-gp expression of vehicle treated CEM/VLB$_{100}$ cells. Black bars represent P-gp levels in whole cell lysate. White bars represent the amount of P-gp present in crude membrane extracts. A representative Western blot for P-gp and alpha tubulin expression with increasing doses of CBD and THC are shown. Curcumin was employed as a positive control for down-regulation of membrane associated P-gp. d.f. = 4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

including the CCRF-CEM cells [31]. Interestingly, it was shown that CCRF-CEM cells contain relatively high levels of functional CB_1 and CB_2 receptors, however, the cytotoxic action of THC on these cells could not be prevented by co-administration of CB_1 or CB_2 receptor antagonists. The exact mechanism responsible for the cytotoxic effects of cannabinoids on CCRF-CEM cells remains elusive. It may involve vanilloid receptors (VR1) [32], the manipulation of membrane lipid rafts [33] or an interaction with an intracellular target. Assuming that cannabinoids act intracellularly to engender their cytotoxic actions in these cell lines, the finding that the drug-resistant cell line (CEM/VLB_{100}) was as sensitive to the antiproliferative actions of cannabinoids as the drug-sensitive cell line (CCRF-CEM) provides superficial evidence that cannabinoids are not substrates for P-gp. Future studies could address the possibility of whether cannabinoids are substrates for P-gp in more detail.

The finding that short-term exposure (1 h) to the plant-derived cannabinoids (CBN, CBD and THC) or the synthetic cannabinoid receptor agonist (WIN) does not increase the intracellular accumulation of Rh123 conflicts with a previous report that a similar concentration of CBN and CBD, but not THC, increases Rh123 accumulation in a *MDR1*-transfected mouse cell line [19]. It is unclear what would account for this contradictory result as our positive control, the potent P-gp inhibitor PSC833, completely reversed the Rh123 accumulation deficit observed in our MDR cells. In addition, Rh123 fluorescence was only measured within the viable cell population, as early apoptotic cells were excluded on the basis of propidium iodide staining and forward and side scatter. Furthermore, our observations were consistent between human MDR cells and *MDR1*-transfected murine cells, ruling out the possibility that the conflicting findings could be attributable to different post-translational modification of P-gp between mouse and human cell lines.

In addition to looking at the direct effect of cannabinoids on P-gp efflux activity using short duration Rh123 accumulation assays (1 h), we examined the effect of CBD and THC on vinblastine cytotoxicity over 72 h. The potency of vinblastine was enhanced several fold in the presence of both CBD and THC. Having demonstrated that these cannabinoids do not directly modulate P-gp activity, we sought to explore whether this increased potency of vinblastine resulted from a reduction in P-gp expression. We first observed that 72 h pre-exposure to CBD or THC produced an increase in intracellular accumulation of Rh123. In addition, Western blot analysis showed that 72 h exposure to THC or CBD reduced P-gp expression in the cell membrane with the magnitude of effect being comparable to that of a known inhibitor of P-gp expression, curcumin [16,17]. Furthermore, cannabinoid treatment induced a greater reduction in P-gp levels in whole cell lysates when compared to membrane extracts. Unlike the cannabinoids, curcumin decreased P-gp expression solely at the cell membrane. This is consistent with previous studies showing curcumin extracts reduce P-gp in the plasma membrane of cervical carcinoma cells [16,17]. The finding that the level of the housekeeping protein, alpha tubulin, was unchanged in response to cannabinoid treatment indicates that the cannabinoid effect observed on P-gp is unlikely to reflect a global reduction in protein synthesis. Taken together, these findings show that 72 h exposure of drug-resistant cells to the cannabinoids, CBD and THC, selectively decreases P-gp expression, with the functional consequence of increased intracellular substrate accumulation and an increased cytotoxic potency of vinblastine.

The observation that cannabinoids reduce P-gp expression with a greater magnitude of effect in whole cell lysates than in a crude membrane preparation implies that cannabinoids also decrease the expression of intracellularly located P-gp. Although P-gp expressed in the plasma membrane appears most important to conferring MDR [34], evidence suggests that its location at intracellular sites (e.g. Golgi apparatus and intracytoplasmic vesicles) may also assist in the sequestration and transport of anti-cancer drugs away from their intracellular targets [35,36]. Interestingly, the global cellular reduction in P-gp following 72 h cannabinoid exposure encompasses the time-frame whereby newly synthesized P-gp is transported from the intracellular compartment to the plasma membrane [34]. Assessment of the relative contribution of plasma membrane versus intracellularly located P-gp to chemosensitization promoted by cannabinoids could be investigated in future studies. Furthermore, cannabinoid-induced alterations in intracellular trafficking of P-gp to the cell membrane could also be addressed.

It has been argued that selectively decreasing induced, but not constitutive P-gp expression is a more attractive therapeutic target than direct inhibition of P-gp efflux activity [18]. Selectively preventing induction of expression in tumour cells would eliminate the adverse pharmacokinetic effects often associated with direct inhibition of P-gp in constitutively expressing tissues responsible for the metabolism and excretion of cytotoxic drugs. It has been proposed that Et-743 may act through such a selective mechanism [37], however, exactly how compounds such as Et-743 and curcumin decrease P-gp expression remains to be elucidated. Similarly, the mechanisms responsible for cannabinoid-induced P-gp down-regulation require further investigation. Exploring the mechanisms responsible for such a reduction in P-gp expression may reveal novel therapeutic targets and aid in the creation of more potent drugs that can decrease induced P-gp levels in tumour cells.

It is possible that cannabinoid receptors mediate the effects of THC and CBD on P-gp expression. A prior study has shown that CB_1 and CB_2 receptors are present on CCRF-CEM cells [31]. THC binds both CB_1 and CB_2 receptors to elicit its actions, whereas CBD only weakly binds these receptors [38]. However, CBD may indirectly activate cannabinoid receptors by enhancing the levels of the endocannabinoid, anandamide, in tumour cells [39]. Consistent with this, CBD inhibits the enzyme responsible for anandamide hydrolysis, fatty acid amide hydrolase, and also inhibits the putative anandamide membrane transporter [40]. Further, cannabinoids decrease the expression of other membrane-bound proteins, such as nerve growth factor Trk and prolactin receptors in breast cancer cell lines, an effect initially mediated by CB_1 receptors, followed by the involvement of the cAMP/protein kinase pathway and of mitogen-activated protein kinase (MAPK) [41]. Interestingly, it has also been reported that cannabinoids modulate nuclear transcription factor levels through the inhibition of the extracellular signal regulated kinase (ERK)

pathway in cells of immune origin [42]. Future investigations could investigate whether cannabinoid receptors mediate cannabinoid-induced P-gp down-regulation and what intracellular signaling pathways are involved. Further, they could investigate whether the observed decrease in P-gp expression results from transcriptional down-regulation of *MDR1* or a disruption in translation of the protein.

Cannabinoid compounds are commonly used as palliative care agents in reducing the adverse effects of conventional chemotherapies and cancer-related pain [43]. Often patients are prescribed with formulations that contain THC-alone (e.g. Marinol®) as these are legally available in some countries for the treatment of chemotherapy-induced nausea and vomiting [24]. A recent development in Canada is the introduction of the sublingual spray, Sativex®. This novel formulation can be prescribed to neuropathic pain sufferers and contains a unique combination of both THC and CBD. Apart from these new formulations, patients may administer cannabinoids by smoking the plant, which contains abundant levels of THC, CBD, and to a lesser extent, other cannabinoids [25]. Interestingly, blood THC concentrations after acute dosing have been reported to increase in a dose-dependent manner approaching a maximal plasma concentration of 1 μM [44]. Concentrations of the cannabinoids used here encompass these levels, although, only a modest effect of cannabinoids on P-gp expression was observed at concentrations that are likely to be attained in vivo (0.1–1 μM). While it is possible that such effects observed here may translate to the clinical situation, future research is needed to better characterise the effects of cannabinoids on MDR, in other cell lines as well as in in vivo animal models.

In conclusion, cannabinoids do not directly inhibit the transport function of P-gp. Rather, prolonged exposure to plant-derived compounds appears to modestly decrease P-gp expression, with a concomitant increase in sensitivity of MDR cells to P-gp substrates, including an anticancer agent. These results suggest that cannabinoids might be safely administered to cancer patients that have developed P-gp mediated resistance to chemotherapeutic agents. However, future preclinical studies are needed to further characterise the effects of cannabinoids on MDR in vitro and in vivo.

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REFERENCES

[1] De Petrocellis L, Cascio MG, Di Marzo V. The endocannabinoid system: a general view and latest additions. Br J Pharmacol 2004;141:765–74.
[2] Smith PF. Medicinal cannabis extracts for the treatment of multiple sclerosis. Curr Opin Investig Drugs 2004;5:727–30.
[3] Karst M, Salim K, Burstein S, Conrad I, Hoy L, Schneider U. Analgesic effect of the synthetic cannabinoid CT-3 on chronic neuropathic pain: a randomized controlled trial. J Am Med Assoc 2003;290:1757–62.
[4] Martin BR, Wiley JL. Mechanism of action of cannabinoids: how it may lead to treatment of cachexia, emesis, and pain. J Support Oncol 2004;2:305–16.
[5] Arceci RJ. Clinical significance of P-glycoprotein in multidrug resistance malignancies. Blood 1993;81:2215–22.
[6] Hayes JD, Wolf CR. Molecular mechanisms of drug resistance. Biochem J 1990;272:281–95.
[7] Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 1989;58:137–71.
[8] Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 1987;84:265–9.
[9] Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, et al. Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst 1989;81:116–24.
[10] Campos L, Guyotat D, Archimbaud E, Calmard-Oriol P, Tsuruo T, Troncy J, et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. Blood 1992;79:473–6.
[11] Leith CP, Kopecky KJ, Chen IM, Eijdems L, Slovak ML, McConnell TS, et al. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood 1999;94:1086–99.
[12] Cornwell MM, Pastan I, Gottesman MM. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem 1987;262:2166–70.
[13] Twentyman PR, Bleehen NM. Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporine. Eur J Cancer 1991;27:1639–42.
[14] Warner E, Hedley D, Andrulis I, Myers R, Trudeau M, Warr D, et al. Phase II study of dexverapamil plus anthracycline in patients with metastatic breast cancer who have progressed on the same anthracycline regimen. Clin Cancer Res 1998;4:1451–7.
[15] Ross DD. Modulation of drug resistance transporters as a strategy for treating myelodysplastic syndrome. Best Pract Res Clin Haematol 2004;17:641–51.
[16] Anuchapreeda S, Leechanachai P, Smith MM, Ambudkar SV, Limtrakul PN. Modulation of P-glycoprotein expression and function by curcumin in multidrug-resistant human KB cells. Biochem Pharmacol 2002;64:573–82.
[17] Limtrakul P, Anuchapreeda S, Buddhasukh D. Modulation of human multidrug-resistance MDR-1 gene by natural curcuminoids. BMC Cancer 2004;4:13–8.
[18] Jin S, Gorfajn B, Faircloth G, Scotto KW. Ecteinascidin 743, a transcription-targeted chemotherapeutic that inhibits MDR1 activation. Proc Natl Acad Sci USA 2000;97:6775–9.
[19] Molnar J, Szabo D, Pusztai R, Mucsi I, Berek L, Ocsovszki I, et al. Membrane associated antitumor effects of crocine-, ginsenoside- and cannabinoid derivates. Anticancer Res 2000;20:861–7.
[20] Beck WT, Mueller TJ, Tanzer LR. Altered surface membrane glycoproteins in Vinca alkaloid-resistant human leukemic lymphoblasts. Cancer Res 1979;39:2070–6.
[21] Hill AB, Beck WT, Trent JM. Cytogenetic and molecular characterization of tumors in nude mice derived from a multidrug-resistant human leukemia cell line. Cancer Res 1988;48:393–8.

[22] Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther 2002;1:417–25.
[23] Bebawy M, Morris MB, Roufogalis BD. Selective modulation of P-glycoprotein-mediated drug resistance. Br J Cancer 2001;85:1998–2003.
[24] Adams IB, Martin BR. Cannabis: pharmacology and toxicology in animals and humans. Addiction 1996;91: 1585–614.
[25] de Meijer EP, Bagatta M, Carboni A, Crucitti P, Moliterni VM, Ranalli P, et al. The inheritance of chemical phenotype in *Cannabis sativa* L. Genetics 2003;163:335–46.
[26] Bifulco M, Di Marzo V. Targetting the endocannabinoid system in cancer therapy: a call for further research. Nat Med 2002;8:547–50.
[27] Guzman M. Cannabinoids: potential anticancer agents. Nat Rev Cancer 2003;3:745–55.
[28] Kogan NM. Cannabinoids and cancer. Mini Rev Med Chem 2005;5:941–52.
[29] McKallip RJ, Lombard C, Fisher M, Martin BR, Ryu S, Grant S, et al. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. Blood 2002;100:627–34.
[30] Munson AE, Harris LS, Friedman MA, Dewey WL, Carchman RA. Antineoplastic activity of cannabinoids. J Natl Cancer Inst 1975;55:597–602.
[31] Powles T, te Poele R, Shamash J, Chaplin T, Propper D, Joel S, et al. Cannabis-induced cytotoxicity in leukemic cell lines: the role of the cannabinoid receptors and the MAPK pathway. Blood 2005;105:1214–21.
[32] Maccarrone M, Lorenzon T, Bari M, Melino G, Finazzi-Agro A. Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. J Biol Chem 2000;275:31938–45.
[33] Sarker KP, Maruyama I. Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. Cell Mol Life Sci 2003;60:1200–8.
[34] Fu D, Bebawy M, Kable EP, Roufogalis BD. Dynamic and intracellular trafficking of P-glycoprotein-EGFP fusion protein: implications in multidrug resistance in cancer. Int J Cancer 2004;109:174–81.
[35] Molinari A, Calcabrini A, Meschini S, Stringaro A, Crateri P, Toccacieli L, et al. Subcellular detection and localization of the drug transporter P-glycoprotein in cultured tumor cells. Curr Protein Pept Sci 2002;3:653–70.
[36] Rajagopal A, Simon SM. Subcellular localization and activity of multidrug resistance proteins. Mol Biol Cell 2003;14:3389–99.
[37] Friedman D, Hu Z, Kolb EA, Gorfajn B, Scotto KW. Ecteinascidin-743 inhibits activated but not constitutive transcription. Cancer Res 2002;62:3377–81.
[38] Pertwee R. Pharmacology of CB1 and CB2 receptors. Pharmacol Ther 1997;74:129–80.
[39] Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio MG, Cosenza V, et al. Possible endocannabinoid control of colorectal cancer growth. Gastroenterology 2003;125: 677–87.
[40] Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, et al. Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 2001;134:845–52.
[41] Melck D, Rueda D, Galve-Roperh I, De Petrocellis L, Guzman M, Di Marzo V. Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells. FEBS Lett 1999;463:235–40.
[42] Faubert BL, Kaminski NE. AP-1 activity is negatively regulated by cannabinol through inhibition of its protein components, c-fos and c-jun. J Leukoc Biol 2000;67:259–66.
[43] Walsh D, Nelson KA, Mahmoud FA. Established and potential therapeutic applications of cannabinoids in oncology. Support Care Cancer 2003;11:137–43.
[44] Azorlosa JL, Heishman SJ, Stitzer ML, Mahaffey JM. Marijuana smoking: effect of varying delta 9-tetrahydrocannabinol content and number of puffs. J Pharmacol Exp Ther 1992;261:114–22.