

Fatty acid amide hydrolase (FAAH) inhibitor PF-3845 reduces viability, migration and invasiveness of human colon adenocarcinoma Colo-205 cell line: an *in vitro* study*

Andrzej Wasilewski¹, Urszula Krajewska², Katarzyna Owczarek¹, Urszula Lewandowska¹ and Jakub Fichna¹✉

¹Department of Biochemistry, Faculty of Medicine and ²Department of Pharmaceutical Biochemistry and Molecular Diagnostics, Faculty of Pharmacy, Medical University of Lodz, Łódź, Poland

Earlier reports suggest that the endocannabinoids may play a role of endogenous tumor growth modulators. In this study, we investigated whether inhibition of the enzymes involved in the synthesis and degradation of endocannabinoids may reduce colorectal cancer cell invasion and migration. The human colon adenocarcinoma Colo-205 cells were incubated with PF-3845, JZL-184 and RHC-80267 (fatty acid amide hydrolase (FAAH), mono- (MAGL) and diacylglycerol lipase (DAGL) inhibitors, respectively) for 48 h. The MTT colorimetric assay was performed to quantify cell viability. Next, Colo-205 cells were incubated with PF-3845 alone or with PF-3845 together with selected antagonists: AM 251, AM 630, SB 366791, RN 1734 and G-15 (CB₁, CB₂, TRPV1, TRPV4 and GPR30 antagonists, respectively). Western blot assay was applied to identify the changes in CB₁ and CB₂ receptor expression. Migration and invasion assays were employed to characterize the effect of PF-3845 on colorectal cancer cell invasion. We found that of all the inhibitors used, the FAAH inhibitor PF-3845 reduced the Colo-205 cell line viability the most effectively (IC₅₀=52.55 μM). We also showed that the effect of decreased cell viability was enhanced when Colo-205 cells were incubated with PF-3845 and RN-1734, a TRPV4 antagonist (IC₅₀=30.54 μM). Western blot assay revealed significantly decreased CB₁ receptor expression levels, while CB₂ expression was increased in response to PF-3845 when compared to control. Furthermore, PF-3845 inhibited migration and invasion of Colo-205 cell line. These results suggest that pharmacological inhibition of FAAH and consequent enhancement of the endocannabinoid levels may reduce the colorectal cancer growth and progression.

Key words: cannabinoid receptors, Colo-205, colorectal cancer, invasion, migration

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✉ e-mail: jakub.fichna@umed.lodz.pl

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Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CRC, colorectal cancer; DAGL, diacylglycerol lipase; EA, ethanolamine; FAAH, fatty acid amide hydrolase; GPER, G protein-coupled estrogen receptor 1; GPR30, G protein-coupled receptor 30; MAGL, monoacylglycerol lipase; NAPE, N-arachidonoyl phosphatidylethanolamine; PLD, phospholipase D; TRPV1, transient receptor potential vanilloid type 1

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide, and constitutes about 10% of all diagnosed cancers (Steward *et al.*, 2014). There are over 1.4 million new cases of CRC worldwide each year, of which over 50 % are found in developed countries; it is also estimated that there are almost 700 000 deaths from CRC worldwide. Although various therapeutic strategies have been used in the treatment of CRC, including newly developed chemotherapeutic agents and monoclonal antibodies, often combined with partial surgical resection of colon, CRC still carries poor prognosis (Kelly *et al.*, 2005). Thus, the real endeavor is to find new therapeutic agents providing more effective treatment.

The global data suggest that cannabinoids may inhibit tumor growth both *in vitro* and in animal tumor models. It has been demonstrated that natural and synthetic exogenous cannabinoids, as well as endocannabinoids inhibit tumor growth and progression of colon, breast, prostate and thyroid cancer, and leukemia by cytotoxic or cytostatic effects, induction of apoptosis, or inhibition of neoangiogenesis (Bifulco *et al.*, 2006). The endocannabinoid system consists of “classical” CB₁ (Matsuda *et al.*, 1990; Gerard *et al.*, 1991) and CB₂ (Munro *et al.*, 1993), and “non-classical” receptors, such as a transient receptor potential vanilloid type 1 (TRPV1) and type 4 (TRPV4), and a G protein-coupled receptor 30 (GPR30), also known as a G protein-coupled estrogen receptor 1 (GPER), as well as their endogenous ligands and metabolizing enzymes (Pertwee 2006).

The two most studied endocannabinoids are anandamide (AEA) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995). Anandamide is mainly synthesized from its membrane phospholipid precursor N-arachidonoyl phosphatidylethanolamine (NAPE) by phospholipase D (NAPE-PLD) (Liu *et al.*, 2008). Termination of AEA signaling occurs through a time- and temperature-dependent uptake mechanism that controls its cellular trafficking and metabolism (Chicca *et al.*, 2012). Anandamide is transported into the cell *via* a cell membrane carrier-mediated transporter and subsequently undergoes a rapid and almost complete intracellular degradation by fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (EA) (Fowler, 2013; Day *et al.*, 2001). 2-arachidonoylglycerol is synthesized from membrane phospholipids *via* sequential activation of phospholipase Cβ and diacylglycerol lipase (DAGL), and degraded by a monoacylglycerol lipase (MAGL).

The aim of our study was to investigate the effect of selected inhibitors of the enzymes involved in the synthesis (RHC-80267) and degradation (JZL-184 and PF-3845) of AEA and 2-AG, on Colo-205 cell viability. Based on the data obtained in the *in vitro* MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay, the FAAH inhibitor PF-3845 ($IC_{50}=52.55 \mu\text{M}$) was chosen for further analysis (Ahn *et al.*, 2009). Next, we assessed the IC_{50} values in cells treated with PF-3845 alone, and co-incubated with selected cannabinoid receptor antagonists (AM 251, AM 630, SB 366791, RN 1734 and G-15 for CB_1 , CB_2 , TRPV1, TRPV4 and GPR30, respectively). To explore the mechanisms of action of PF-3845 in Colo-205 cell viability, we characterized the expression of CB_1 and CB_2 receptors by Western blot analysis. We also examined the effect of PF-3845 on Colo-205 cell migration and invasiveness. Collectively, our results show for the first time that PF-3845 may play a crucial role in inhibiting viability, migration and invasiveness of the Colo-205 cell line. Therefore, PF-3845 has the potential to be an efficient anti-cancer drug in therapeutic strategies for colorectal cancer.

MATERIALS AND METHODS

Culture conditions. Human colon adenocarcinoma Colo-205 cells were purchased from the European Collection of the Cell Cultures (ECACC). Colo-205 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 1%

non-essential amino acids. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. All experiments were carried out between passages 5 and 15.

Reagents. Enzyme inhibitors: PF-3845, JZL-184 and RHC-80267, selective for FAAH, MAGL, and DAGL, respectively (Fig. 1), the CB_1 and CB_2 receptor antagonists: AM 251 and AM 630, and the TRPV1, TRPV4 and GPR30 antagonists: SB 366791, RN 1734 and G-15, were purchased from Tocris Bioscience (Ellisville, MO). Inhibitors and antagonists were dissolved in dimethyl sulfoxide (DMSO) and then diluted to the final concentration with a serum-free medium. DMSO was purchased from Sigma-Aldrich, Inc. (St. Louis, Mo, USA). Cell culture media and supplements were purchased from Gibco (Life Technologies), except for amphotericin B (Biochrom AG, Berlin, Germany). Antibodies to CB_1 (sc-20754), and CB_2 (sc-25494) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and β -actin (A0545) was purchased from Sigma-Aldrich.

MTT assay. The effect of compounds on Colo-205 cell viability was assessed by the MTT assay as described previously (Hansen *et al.*, 1989). The assay principle is based on the fact that mitochondrial dehydrogenases of metabolically active cells reduce the tetrazolium ring to an insoluble MTT formazan crystals. The resulting purple solution is spectrophotometrically measured and cell viability can be assessed. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen, Paisley, UK), 4 mM L-glutamine, and antibiotics (50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin), and 1% non-essential amino acids. Exponentially growing colorectal cancer cells were seeded at 9.3×10^3 /well in a 96-well plate (Nunc, Roskilde, Denmark). Stock solutions of the analyzed compounds were freshly prepared in DMSO and diluted with complete culture medium to obtain the concentration range from 10^{-7} to 10^{-3} M. The final concentration of DMSO in the medium did not exceed 0.02%. Cells were exposed to the test compound for 46 h, then MTT reagent (Sigma, St. Louis, USA) was added (5 mg/mL PBS) and incubation was continued for 2 h. MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH 4.7 and absorbance was read at 570 nm with a Victor 3-plate reader (Perkin-Elmer, Turku, Finland). For control, cells were grown in the absence of compounds, but with their respective vehicle. Values of IC_{50} (the concentration of tested compounds required to reduce colon cancer cell survival fraction to 50% of control) were used as a measure of cellular sensitivity to a given treatment. Data points represent means from at least three independent experiments performed in triplicate.

Western blot analysis. The cells were lysed using a RIPA buffer (mammalian cell lysis kit, MCL-1; Sigma-Aldrich) with a protease inhibitor cocktail and centrifuged at $14000 \times g$ for 20 min at 4°C supplemented with phosphatase and protease inhibitors. Cell lysates were stored at -80°C until analysis. The samples protein concentration was assayed using the Bradford method. Aliquots of the lysates (30 μg of protein) were boiled for 5 min, denatured and separated by 12% SDS-polyacrylamide gel electrophoresis. Next, a Bio-Rad Trans-Blot system was used to transfer the proteins to a PVDF membrane. After the transfer, the membrane was blocked with 5% non-fat milk in Tris buffered saline containing 0.1% Tween 20 (TBS-T) and then incubated for two hours. The membrane was then washed in TBS-T and hybridized with primary rabbit CB_1 and CB_2 polyclonal antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), which were diluted to a suitable concentration (1:200 –

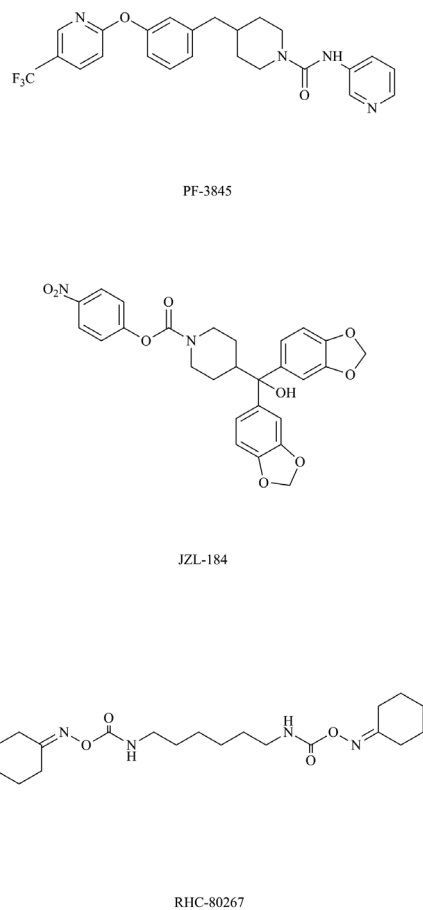


Figure 1. Chemical formulas of the tested inhibitors: PF-3845, JZL-184 and RHC-80267.

1:500) in TBS-T, for 16 h. Additionally, the membranes were incubated with a goat polyclonal antibody specific for β -actin (1:100 000 dilution) to use as a loading control. The membrane was washed six times with TBS-T and incubated with appropriate peroxidase-conjugated secondary antibody, and the protein bands were visualized using enhanced chemiluminescence Western blotting detection kit (Super Signal West Femto Reagent, Thermo Fisher Scientific, Rockford, IL, USA).

Migration assay. Cell migration assay was performed using the BD Falcon™ 24-Multiwell Insert plate system (Becton Dickinson, Bedford, MA, USA), as described previously, with a slight modification (Lewandowska *et al.*, 2013). Colo-205 cells were resuspended in a serum-free medium with or without PF-3845 (0–100 μ M) and plated onto the upper chamber at the densities of 1×10^5 per 500 μ L. The lower chamber contained medium with 10% FBS as a chemoattractant. The chambers were incubated for 48 h. After incubation, the non-migrated cells in the upper chamber were gently scraped away and adherent cells present on the lower surface of the insert were stained with crystal violet, photographed, and counted using the NIH ImageJ analysis software.

Invasion assay. Invasion studies were conducted using the Matrigel BM matrix assay developed for measurement of tumor cell invasiveness. We used BioCoat Matrigel invasion chambers (24-well cell culture inserts containing an 8.0- μ m PET membrane with a uniform layer of Matrigel [Becton Dickinson, Bedford, MA]). Cells were resuspended in a serum-free medium with or without PF-3845 (0–100 μ M) and plated onto the upper chamber at the densities of 1×10^5 per 500 μ L. The lower chamber contained medium with 10% FBS as a chemoattractant. The chambers were incubated for 72 h. After incubation, the non-migrated cells in the upper chamber were gently scraped away and adherent cells present on the lower surface of the insert were stained with crystal violet, photographed, and counted using the NIH ImageJ analysis software.

Statistical analysis. Data are presented as means \pm standard deviation. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni *t*-test for multiple comparisons. The significance between two groups was determined using *t*-test. Statistical analyses were performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences resulting in a *p*-value of 0.05 or less were considered to be significant.

RESULTS

PF-3845, but not JZL-184 and RHC-80267, significantly decreased the Colo-205 cell viability

In our study, we first determined the influence of selected inhibitors: PF-3845, JZL-184 and RHC-80267 (selective for FAAH, MAGL and DAGL, respectively) on Colo-205 cell viability. PF-3845, at concentrations lower than 10 μ M, had an inconsiderable effect (viability reduced by 7.73% at 10 μ M concentration) versus control, while it had a significant influence on Colo-205 cell viability ($p < 0.001$) at the concentration of 100 μ M (89.90% decrease in viability) (Fig. 2).

A significant effect for JZL-184 ($p < 0.001$) has been observed only at the concentration of 1000 μ M, with reduced cell viability to 43.16%. We did not observe any effect of JZL-184 at concentrations 0.1, 1, 10, and 100 μ M on the Colo-205 viability. Similarly, Colo-205 cell

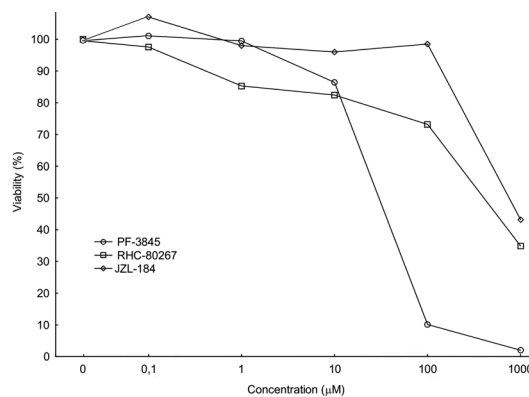


Figure 2. Survival curves for Colo-205 exposed to the tested compounds: PF-3845, JZL-184 and RHC-80267.

Cells (9.3×10^3 cells/well) were seeded in 96-well culture plates. After 24 h, the cells were treated with PF-3845, JZL-184 or RHC-80267 (0, 0.1, 1.0, 10, 100, 1000 μ M) for another 48 h. Cell viability was measured by MTT assay. Each point represents the mean of 5–10 independent determinations. Standard deviations were excluded for clarity and did not exceed 12% of the mean value for each point.

viability did not clearly decrease in response to RHC-80267 at the concentrations lower than 10 μ M. The effect of RHC-80267 reached a statistically significant decrease at 100 and 1000 μ M ($p < 0.001$), with reduction in cell viability to 73.40 and 34.88%, respectively.

Based on the MTT test results and calculated IC_{50} values for inhibitors: 52.55, 900.89 and 638.95 μ M for PF-3845, JZL-184 and RHC-80267, respectively, the former was selected for further studies as the most efficient in decreasing the Colo-205 cell viability. Thus, we next compared the IC_{50} values in cells treated with PF-3845 alone, and PF-3845 with selected receptor antagonists (Fig. 3). The calculated IC_{50} values were lower when cells were treated with PF-3845+antagonist than with PF-3845 alone. Namely, PF-3845 co-incubated with a TRPV4 antagonist RN 1734 displayed the highest potency to reduce Colo-205 cell viability, with an IC_{50} value of 30.54 μ M (*vs.* IC_{50} =51.38 μ M for PF-3845 alone). The IC_{50} values for PF-3845 + AM 251, PF-3845+SB-366791, and PF-3845+AM-630 were 37.51, 38.49, and 40.20 μ M, respectively. On the contrary, PF-3845+G-15 (GPR30 antagonist) exhibited slightly

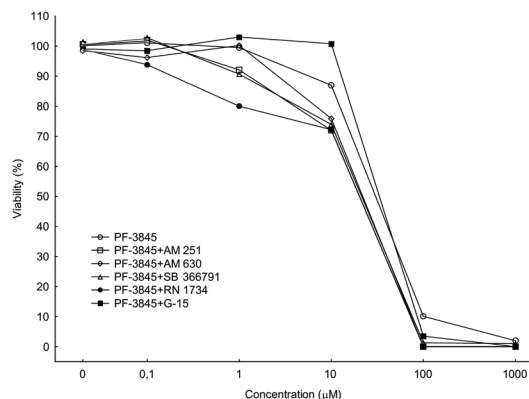


Figure 3. Survival curves for Colo-205 exposed to FAAH inhibitor PF-3845 alone or co-incubated with selected antagonists: AM 251 (CB1 receptor antagonist), AM 630 (CB2), SB 366791 (TRPV1), RN 1734 (TRPV4) and G-15 (GPR30).

Each point represents mean of 5 independent determinations. Standard deviations were excluded for clarity and did not exceed 12% of the mean value for each point.

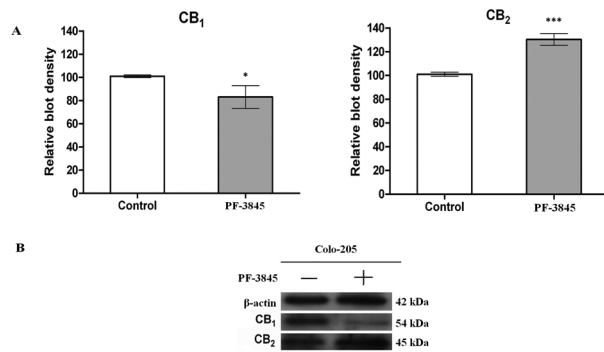


Figure 4. Effect of PF-3845 on CB₁ and CB₂ receptor expression in the Colo-205 cells.

(A) Cells were treated with PF-3845 for 48 h and subjected to Western blot analysis. PF-3845 decreased expression of CB₁ and increased expression of CB₂ receptor. (B) β-actin in the cell lysate is shown as control. Each blot is representative of at least three other blots. Density values of the bands are graphically expressed relative to control. Data are shown as mean ± S.D. (n=4). Significance of differences between means: * $p < 0.05$ versus control; *** $p < 0.001$ versus control.

higher IC₅₀ value (55.89 μM) when compared with IC₅₀ of PF-3845 alone (IC₅₀ = 51.38 μM).

Expression of CB₁ receptors decreased, while CB₂ increased in the Colo-205 cells

Anandamide is a known CB₁ and CB₂ receptor agonist (Reggio *et al.*, 2000). To examine the effect of the inhibition of FAAH by PF-3845 on the expression of CB₁ and CB₂ receptors in the Colo-205 cells, Western blot analysis was used. As shown in Fig. 4A and 4B, when the cells were treated with PF-3845 at the concentration of 52.55 μM (IC₅₀ value determined for PF-3845 alone in the previous stage), a significant ($p < 0.05$) decrease was observed in expression of the CB₁ receptor. On the contrary, CB₂ expression level significantly increased ($p < 0.001$) in response to PF-3845 when compared with the untreated control. Taken together, these results demonstrate that PF-3845 affects expression of the key receptors of endocannabinoid pathway.

PF-3845 significantly reduces migration and invasiveness of the Colo-205 cells

To investigate the activity of PF-3845 against tumor metastasis, we investigated its effects on migration of the Colo-205 cells. As illustrated in Fig. 5, the data from the migration assay indicated that migration of the Colo-205 cells was clearly inhibited by PF-3845 in a concentration-dependent manner (Fig. 5A and 5B). Suppression of the Colo-205 migration reached 49.57, 81.73, 96.4 and 100% versus control at PF-3845 concentrations of 25 μM, 50 μM, 75 μM and 100 μM, respectively.

The loss of cell-cell adhesion capacity allows tumor cells to dissociate from the primary tumor mass leading to the spread of metastatic cancers. In the next stage, we decided to test whether PF-3845 displays the potency to reduce colorectal cancer cell invasion. An invasion assay in Matrigel-coated transwell chamber indicated that PF-3845 inhibited invasiveness of the Colo-205 cells (Fig. 6A and 6B). At the lowest concentration tested (25 μM), PF-3845 was not efficient in inhibiting Colo-205 invasiveness and the reduction was only of 5.53% vs. control, whereas at 50, 75 and 100 μM concentrations it reached 71.28%, 88.50%, and 100% versus control, respectively.

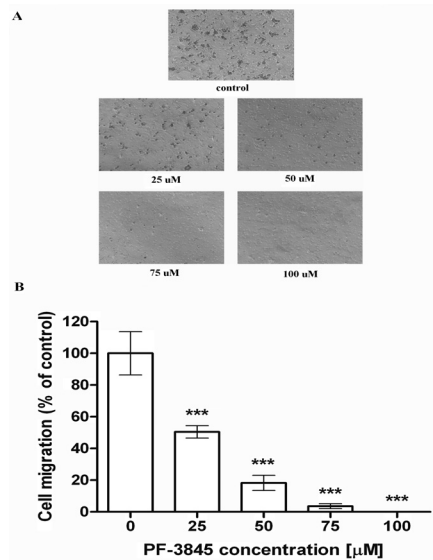


Figure 5. Inhibition of Colo-205 cell migration by PF-3845 in the concentration range of 0 to 100 μM.

Migration studies were conducted using BD Falcon™ 24-Multiwell Insert plate system. Total area of inserts with crystal violet-stained cells was photographed and counted using the NIH ImageJ analysis software. (A) Representative images obtained for Colo-205 are shown above graphs. Initial magnification: x200. (B) Each value on the graph represents the mean value ± S.D., n=3 independent experiments. Significance of differences between means: *** $p < 0.001$ versus control.

DISCUSSION

Here, we have demonstrated that modulation of the activity of enzymes involved in the endocannabinoid turnover translates into cancer cell viability, migration and metastasis. This may have an important implication

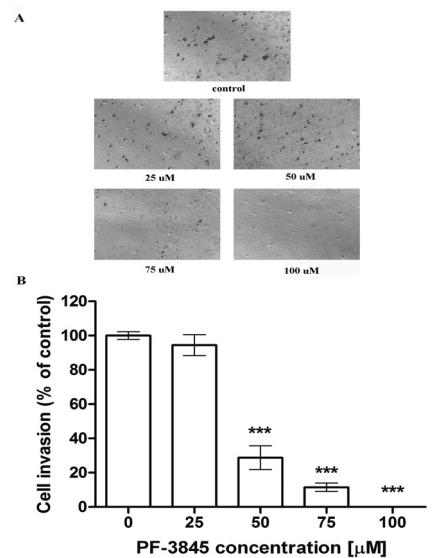


Figure 6. Inhibition of Colo-205 cell invasiveness by PF-3845 in the concentration range of 0 to 100 μM.

Invasion studies were conducted using the Matrigel BM matrix assay developed for measurement of tumor cell invasiveness. Total area of inserts with crystal violet-stained cells was photographed and measured using the NIH ImageJ analysis software. (A) Representative images obtained for Colo-205 are shown above graphs. Initial magnification: x 200. (B) Each value on the graph represents the mean value ± S.D., n=3 independent experiments. Significance of differences between means: *** $p < 0.001$ versus control.

for development of anticancer strategies targeting the endocannabinoid system.

We have observed that the Colo-205 cell viability was reduced after treatment with PF-3845. It is a potent and selective inhibitor of the FAAH enzyme which hydrolyzes AEA into arachidonic acid and EA (Bifulco *et al.*, 2004; De Lago *et al.*, 2006; Endsley *et al.*, 2007). Blocking of the endocannabinoid degradation has been proven to be effective for eradication of other cancer cell types. For example, Matas and coworkers (2007) had observed that the non-selective FAAH inhibitor URB597 induces cell death in neuroblastoma cells. Noteworthy, this effect was obtained by preventing generation of EA from AEA, and not by increasing the AEA level or signaling through the CB receptors. What may be important also for our study, Matas and coworkers (2007) had shown that EA exerts an anti-apoptotic action; therefore, a pharmacologically-induced decrease of EA levels may deteriorate cancer cell viability. Earlier reports indicate that the inhibition of FAAH may induce cell death also by activating the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2)/antioxidant responsive element (ARE) signaling pathway and the heme oxygenase-1 (HO-1) induction and transcription (Ranger *et al.*, 2009). These pathways can be taken into consideration when explaining the anti-cancer action of PF-3845 observed in our study. Of note, we did not observe any statistically significant effect of JZL-184, a MAGL inhibitor, on reduction of the Colo-205 cell viability. This is in contrast to Ye and coworkers (2011) *et al.* who had demonstrated inhibition of the tumor cell growth and invasion via knockdown of MAGL by JZL-184 and siRNA in human CRC cells.

In search for underlying pathways, we have assessed the influence of Colo-205 cell incubation with PF-3845 alone and in the presence of "classical" (CB₁ and CB₂) and "non-classical" (TRPV1, TRPV4, GPR30) cannabinoid receptor antagonists (AM 251, AM 630, SB 366791, RN 1734 and G-15, respectively). Interestingly, we have found that co-incubation with the receptor antagonists, except for G-15, reduced the Colo-205 cell viability, with the highest potency displayed by RN 1734. In contrast, a slight, but not statistically significant antagonizing effect of G-15 was observed. Our data may suggest that the effect of PF-3845 on the Colo-205 cell viability is not necessarily CB₁, CB₂, TRPV1 or TRPV4-dependent. Further studies are needed to verify whether a different receptor type, either "classical" or "non-classical" is involved.

Noteworthy, the higher potency to reduce Colo-205 cell viability by PF-3845 in the presence of the antagonists may be related to a dual action of this inhibitor; namely, PF-3845 inhibits FAAH and may be also involved in blocking of the AEA uptake through actions on the plasma membrane transport protein (Björklund *et al.*, 2014; Day *et al.*, 2001). It is known that AEA activates the TRPV1 capsaicin receptor and leads to an increase of intracellular Ca²⁺-levels (Ralevic *et al.*, 2001). Recently, TRPV1 has been found to contribute to AEA transport into endothelial cells in a Ca²⁺-independent manner and to be involved in the pro-angiogenic effect of AEA in the endothelial cells (Hofmann *et al.*, 2014). Therefore, a complex interplay of the effects mediated by receptor and uptake proteins in the presence of PF-3845 and these protein inhibitors needs to be acknowledged. It has been also shown previously that the increase of cannabinoid receptor expression may lead to activation of the pro-survival signal of the Akt kinase, and as a result, the treatment does not induce apoptosis, unless the Akt activation is blocked (Cudaback *et al.*,

2010). Whether this is the case for PF-3845 and Colo-205, especially in view of the cannabinoid receptor expression data discussed below, remains to be determined.

The study presented here was also designed to estimate the effect of PF-3845 on CB₁ and CB₂ receptor expression level. Here, we found that the CB₁ level decreased, while CB₂ expression was increased in cells incubated with the FAAH inhibitor. There are only a few studies focused on the expression of the cannabinoid receptors in tumor cells. An increased expression of CB₁ and/or CB₂ has been demonstrated in mantle cell lymphoma (Ek *et al.*, 2002; Islam *et al.*, 2003), acute myeloid leukemia (Alberich *et al.*, 2004), breast cancer (Caffarel *et al.*, 2006), and prostate cancer cell lines. Of note, CB₁ receptor expression by tumor cells was associated with poor prognosis in patients with prostate cancer (Cipriano *et al.*, 2013). Downregulation of CB₁ receptor expression was observed in neoplastic epithelial cells from colon cancer biopsies (Cianchi *et al.*, 2008). Jung *et al.* had demonstrated that CB₁ receptor expression was correlated with distant metastasis in CRC, but not with tumor invasion and lymph node metastasis, and CB₁ expression was downregulated as CRC progressed to a highly advanced stage of disease (Jung *et al.*, 2013). Gustafsson *et al.* had shown that high intensity of the CB₁ receptor was correlated with a shorter survival time than with a low CB₁ receptor intensity (Gustafsson *et al.*, 2011). Wang *et al.* had shown that CB₁ expression was silenced in human CRC due to high (77%) methylation of the CB₁ promoter (Wang *et al.*, 2008). To summarize, whether the PF-3845-induced changes in the cannabinoid receptor expression result from adaptation mechanisms upon modulation of the endocannabinoid levels and whether these can be efficiently used in colon cancer treatment needs further investigation.

In this work, we have also investigated the effects of PF-3845 on migration ability of the Colo-205 cell line. We have found that PF-3845 decreases migration of tumor cells by 49.57%, already at the concentration of 25 μM. This correlates with a general observation that cannabinoids are anti-migratory agents in cancer cell lines. For example, Met-fluoro-anandamide (Met-F-AEA), an analog of AEA, caused a CB₁ receptor-dependent antimigratory effect in breast cancer cells. Met-F-AEA inhibited the activity of the GTPase, RhoA, and led to translocation of RhoA to the cytosol, which caused changes in the actin cytoskeleton (Laezza *et al.*, 2008). Another study had shown that cell migration initiated by mast cells was reduced by 2-AG and WIN-55,212-2 in a CB₁ receptor dependent manner (Rudolph *et al.*, 2008). Noteworthy, AEA and HU-210 were shown to block the migration of colon carcinoma cells with low CB₂ receptor expression. On the contrary, as shown for cannabidiol (CBD), its effect on inhibition of the U87 human glioma cell migration was not mediated via the classical CB receptors or a G protein-coupled signaling-related mechanism (Massi *et al.*, 2004; Vaccani *et al.*, 2005).

The invasion capacity of cancer cells determines their penetration power into surrounding tissues, a crucial early step in the metastatic cascade. Here, we investigated the influence of PF-3845 on invasiveness of the Colo-205 cell line and observed that blocking FAAH efficiently inhibits cell invasion. Similarly, another study reported that increase in the 2-AG levels significantly inhibits invasion of the human androgen-independent prostate cancer cells, PC-3 and DU-145. The effect is comparable to that of WIN55,212-2 and (R)-(+)-methanandamide,

which decrease PC-3 and DU-145 cell invasion in a CB₁-dependent manner (Nithipatikom *et al.*, 2004).

Interestingly, PF-3845 was efficient in inhibiting migration of the Colo-205 cells at non-cytotoxic concentrations, whereas the inhibition of invasiveness was observed at concentrations equal to IC₅₀ or higher. The clinical significance of this observation needs further investigation.

CONCLUSION

Our study demonstrates that the FAAH blocker PF-3845, but not MAGL and DAGL blockers JZL-184 and RHC-80267, effectively decreases viability, migration and invasiveness of the Colo-205 cell line. These results suggest that modulation of the endocannabinoid turnover through inhibition of their hydrolysis could be a potential anticancer strategy. However, more studies are needed to clarify long-term effects of FAAH blockers on cancer cells and translating these into clinical conditions.

Author contributions

Wasilewski A, Krajewska U, Lewandowska U and Fichna J conceived and designed the experiments; Wasilewski A, Krajewska U, Owczarek K and Lewandowska U performed the experiments; Wasilewski A, Krajewska U, Lewandowska U and Fichna J analyzed the data; all authors drafted the article and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the paper.

Conflicts of interest

The authors declare no conflict of interest.

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