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The CB1 receptor antagonist rimonabant controls cell viability and ascitic tumour growth in mice

Anna Maria Malfitano [a], Chiara Laezza [b], Mario Galgani [b], Giuseppe Matarese [b], Alba D'Alessandro [a], Patrizia Gazzerro [a], Maurizio Bifulco [a,*]

[a] Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, Salerno, Italy
[b] Dipartimento Biologia e Patolologia Cellulare e Molecolare and Institute of Endocrinology and Experimental Oncology, IEOS, CNR, Naples, Italy

Endocannabinoid Research Group

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ABSTRACT

Emerging findings suggested the efficacy of the cannabinoid CB1 receptor antagonist rimonabant (SR141716) in several pathological conditions included tumours. In this study we investigated *in vitro* the effects of SR141716 on viability and the molecular pathways of methylcholanthrene-induced fibrosarcoma (Meth-A) cells and *in vivo* its anti-tumour properties in Meth-A-bearing mice. We evaluated *in vitro* the effect of SR141716 on Meth-A cell viability by trypan blue staining assay. Cell cycle progression and apoptosis were assessed by flow cytometry. Protein expression was investigated by Western blot. The anti-tumour efficacy of SR141716 was evaluated *in vivo* monitoring weight increase and survival of Meth-A injected mice. SR141716 affects Meth-A cell viability inducing apoptosis and controls cell cycle progression by modulation of the levels of the cell cycle inhibitor p21waf, cyclins E, D1 and NF-kB molecules. Importantly, SR141716 affects AKT/pFoxO1 pathway which promotes cell survival and regulates the cell cycle. The molecular effects observed are accompanied by reduced COX2 expression and induction of the CB1 receptor expression. Finally, SR141716 was able to reduce the tumour size and prolong animal survival, when administered *in vivo* during tumour growth. Our findings shed light on a novel molecular pathway associated with control of tumour growth by SR141716 and confirm the anti-cancer and anti-inflammatory properties of this drug suggesting its potential applications in the treatment of cancer.

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1. Introduction

Rimonabant (SR141716) (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl pyrazole-3-carboxamide) is a potent and selective cannabinoid CB1 receptor antagonist with food intake inhibiting function and anti-obesity activity [1–7]. It is widely used to investigate the mechanisms involved in the pharmacological effects of cannabinoid agonists, and may exert several intrinsic actions by block of the CB1 receptor induction due to pathophysiological activation of the endocannabinoid system [7–9]. Previous findings showed that SR141716 counteracts most of the anti-tumour effects of anandamide (AEA), suggesting that CB1 receptors are uniquely involved in the effects of this compound [10,11]. Interestingly, besides its antagonist properties, SR141716 exerts also inverse-agonist effects [1,12,13] exhibiting a significant anti-tumour action *in vivo* [10]. In tumour xenografts induced by the subcutaneous injection of rat thyroid cancer KiMol cells in athymic mice, SR141716 exerts a small, although significant, anti-tumour effect on thyroid tumours both *in vitro* and *in vivo* [10]. Other studies reported that SR141716 decreases viability of primary mantle lymphoma cells isolated from tumour biopsies [14], enhances AEA-mediated apoptosis of C6 rat glioma cells [15]. Nonetheless, in C6 cells, SR141716 fails to revert the anti-proliferative effect of CB1 agonists, whereas a combination of cannabinoid and vanilloid (VR) receptor antagonists (SR141716, SR144528 and capsazepine) completely blocks the anti-proliferative effect of AEA [16]. We also showed that SR141716 inhibits human breast cancer cell proliferation, being more effective in highly invasive metastatic MDA MB-231 cells than in less-invasive T47D and MCF-7 cells, effect depending on the presence and expression levels of the CB1 receptor. This effect is not accompanied by apoptosis or necrosis and is characterized by G1/S-phase cell cycle arrest, decreased expression of cyclin D and E and increased levels of cyclin-dependent kinase inhibitor p27KIP1, in addition this effect requires lipid rafts/caveolae integrity to occur. SR141716 exerts a significant anti-proliferative action, *in vivo*, reducing the volume of xenograft tumours induced by MDA-MB-231 injection in mice. On the other hand, at the concentration

* Corresponding author. Tel.: +39 089 969742; fax: +39 089 969602.
E-mail addresses: maubiful@unisa.it, maubiful@unina.it (M. Bifulco).

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range in which we observed anti-proliferative effect in tumour cells, we did not observe genotoxic effects in normal cells [17]. Interestingly, SR141716 used in combination with the CB1 agonist Met-F-AEA, shows synergistic/additive effects in the blockade of human peripheral blood lymphocyte proliferation, this effect is characterized by cell cycle arrest, indeed reduced expression of cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) suggests potential anti-inflammatory action of SR141716 [18]. Moreover, we demonstrated that SR141716 significantly inhibits human colorectal cancer cell (DLD-1, CaCo-2 and SW620) viability [19] altering the cell cycle distribution with G2/M arrest in DLD-1 cells accompanied by induction of mitotic catastrophe. Indeed, in the mouse model of azoxymethane-induced colon carcinogenesis, SR141716 significantly decreases aberrant crypt foci (ACF) formation, which precedes colorectal cancer [20]. Furthermore we recently showed that SR141716 inhibits DLD-1 cell proliferation to the same extent as oxaliplatin and their combination provides a strong synergistic action [21]. Indeed, CB1 inhibition or genetic deletion attenuates the cardiomyopathy and nephropathy induced by chemotherapeutic drugs doxorubicin and cisplatin by modulating oxidative and nitrosative stress and inflammatory response [22–25]. In this study we examined the effects of SR141716 *in vitro* in Meth-A cell line and *in vivo* in Meth-A induced ascites in mice. We showed that SR141716 reduces cell viability of Meth-A cancer cells inducing apoptosis and altering cell cycle progression as revealed by enhanced levels of the cell cycle inhibitor, p21waf and modulation of cyclins E (Cyc E) and D1 (Cyc D1). In addition, we showed for the first time, that the anti-cancer effects of this compound influence the phosporylated kinase protein Akt (pAkt)/FOXO1 pathway and that this phenomenon was accompanied by nuclear factor kappa B (NF-kB) and COX2 reduction. Finally, we observed that SR141716 reduces Meth-A cell growth *in vivo* as indicated by the reduced tumour volume and overrate survival of SR141716-treated mice.

2. Materials and methods

2.1. Drugs

Rimonabant (SR141716, N-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4 dichlorophenyl)-4-methyl-pyrazole-3-carboxamide) was kindly provided by Sanofi-aventis (Montpellier, France) and dissolved in dimethylsulfoxide (DMSO). 2-Methyl-2′-F-anandamide (Met-F-AEA) was purchased from Calbiochem and dissolved in ethanol.

2.2. Cell culture

The transplantable sarcoma Meth-A cells have a well defined immunogenicity and typically produce either solid tumours or liquid ascites when injected s.c. or i.p., respectively [26,27]. Meth-A cells were grown in T25-cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI culture medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and glutamine (2 mM).

2.3. Cell viability assays

Exponentially growing cells (1×10^5) were seeded in 24-well plates. Increasing concentrations of SR141716 (0.3 μM, 1 μM, 3 μM, 10 μM,) and Met-F-AEA (used alone or in combination with SR141716 at equimolar concentrations) were added to the culture. After 24 h and 48 h of incubation, cell were harvested, stained with trypan blue (0.5% solution, Sigma) and counted by hemocytometer.

2.4. Flow cytometry assays

To quantify apoptosis and cell cycle progression, 10^5 Meth-A cells were cultured in the presence and in the absence of SR141716 at the above mentioned concentrations in RPMI 10% FBS for 24 h and 48 h at 37 °C in 24-well plates. To detect apoptosis cells were collected, washed twice with PBS, re-suspended in Annexin V buffer and stained with Annexin V-FITC (Pharmingen, San Diego, CA, USA) for 15 min in the dark at room temperature. Flow cytometry acquisition was performed and data were analysed using Cell Quest software (BD Biosciences, Palo Alto, CA, USA). To analyse cell cycle progression, cells were collected, washed twice with PBS and re-suspended in 300 μl of PBS; 700 μl of 70% ethanol were added slowly to the cells on vortex and kept at −20 °C for 1 h. Propidium iodide (PI) (Sigma, St. Louis, MO) (10 μg ml^{-1}) in PBS containing 100 U ml^{-1} DNase-free RNase was added to the cells; after 15 min at room temperature, cells were subjected to flow cytometric analysis using Summit v4.3 program (Dako Cytomation). Each sample was analysed using 10,000 events corrected for debris and aggregate populations.

2.5. Electrophoresis and immunoblots

Cells were treated with SR141716 at 0.3 μM, 1 μM, 3 μM and 10 μM. After 48 h of incubation, cells were washed twice with PBS, re-suspended in lysis buffer (HEPES 50 mM, NaCl 150 mM, EDTA 50 mM, NaF 100 mM, Na orthovanadate 2 mM, glycerol, $Na_4P_2O_7$ 10 mM and 10% Triton at pH 7.5) and passed through a 23-gauge needle, 10 times before centrifugation at 12,000 × *g* at 4 °C. Supernatants were collected and the protein concentration evaluated by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein extracts (50 mg) were boiled in sample buffer and analysed by electrophoresis in 12% sodium dodecylsulphate polyacrylamide gel. Separated proteins were transferred to nitrocellulose membranes (180 mA at 300 V) for 45 min. The blots were blocked in PBS containing 0.1% Tween-20 and 5% non-fat dry milk for 1 h at room temperature. The filters were then probed overnight with primary antibodies specific for Cyc D1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060), Cyc E (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060), p21waf (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060), protein kinase Akt (Akt) (Cell Signalling Technology, Danvers, MA 01923), pAkt (Ser 473; Cell Signalling), IkB alpha (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060), NF-kB (Cell signalling, Danvers, MA 01923), FOX O1Ser256 (Cell Signalling Technology, Danvers, MA 01923), COX2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060) and CB1 (H-150: sc-20754) (Santa Cruz Biotechnology Inc., Santa Cruz, CA 95060). Immunodetection of specific proteins was carried out with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Bio-Rad, Hercules, CA 94547), using the enhanced chemiluminescence (ECL) system (Amersham, GE Healthcare, Buckinghamshire, UK). Actin as control was used to normalize.

2.6. Animals

Female CB6F1 (BALB/c × C57BL76) (6–7 weeks old) were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved and mice were maintained in accordance to institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized at the University of Naples Medical School Animal Facility for 1 week before injection with cancer cells.

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