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The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Human Breast Cancer Cell Proliferation Through A Lipid Rafts Mediated Mechanism

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Running title: Lipid rafts and anti-proliferative effect of SR141716

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The nonstandard abbreviations used are**:** CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; Met-F-AEA, 2-methyl-arachidonoyl-2'-fluoro-ethylamide; CAs, chromosome aberrations; cdk, cyclin-dependent kinases; MCD, methyl-β-cyclodextrin

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Abstract

The endocannabinoid system has been shown to modulate key cell-signalling pathways involved in cancer cell growth. In this study we show that CB1 cannabinoid receptor antagonist Rimonabant (SR141716) inhibited 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. The SR141716 anti-proliferative effect was not accompanied by apoptosis or necrosis and was characterized by a G1/S phase cell cycle arrest, decreased expression of cyclin D and E and increased levels of cyclin-dependent kinases inhibitor $p27^{KIP1}$. We have also shown that SR141716 exerted a significant anti-proliferative action, *in vivo*, by reducing the volume of xenograft tumors induced by MDA-MB-231 injection in mice. On the other hand, at the concentration range in which we observed the anti-proliferative effect in tumor cells, we did not evidence any genotoxic effect on normal cells. Our data also indicate that the SR141716 anti-proliferative effect requires lipid rafts/caveolae integrity to occur. Indeed, we found that CB1 receptor is completely displaced from lipid rafts in SR141716 treated MDA-MB-231 cells and cholesterol depletion by methyl-beta-cyclodextrin strongly prevented SR141716-mediated anti-proliferative effect. Taken together, our results suggest that SR141716 inhibits human breast cancer cell growth via a CB1 receptor lipid rafts/caveolae-mediated mechanism.

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The "endocannabinoid system", comprising the CB1 and CB2 receptors, their endogenous ligands (endocannabinoids) and the proteins that regulate endocannabinoid biosynthesis and degradation, controls several biological functions, including food intake in animals (Jbilo et al., 2005; Bensaid et al., 2003) and cell proliferation and apoptosis in cancer cells (De Petrocellis et al., 2004). Indeed, recent evidence indicates that endocannabinoids influence the intracellular events controlling the proliferation of numerous types of cancer cells, thereby leading to both *in vitro* and *in vivo* anti-tumor effects (Guzman, 2003). We have previously observed that endocannabinoids inhibit the proliferation of human breast cancer cells by blocking the G0/G1-S phase transition of the cell cycle through interference with cannabinoid CB1 receptor-coupled signal transducing events (De Petrocellis et al., 1998).

It appears that endocannabinoids can act as selective inhibitors of human breast cancer cell proliferation through a growth factor-dependent mechanism (Melck et al., 2000). We have also observed that activation of cannabinoid CB1 receptors can be involved to slow down the growth of breast carcinoma but also to inhibit the metastatic diffusion *in vivo* (Portella et al., 2003; Grimaldi et al., 2006).

It has been previously described that a selective CB1 receptor antagonist, SR141716 but not the CB_2 receptor antagonist counteracts most of the anti-tumor effects of anandamide (AEA), suggesting that CB1 receptors are uniquely involved in the effects of this compound (Grimaldi et al., 2006; Bifulco et al., 2004). Interestingly, besides its antagonist properties SR141716 has also inverse-agonist effects (Rinaldi-Carmona et al., 1994; Hurst et al., 2002) as it can block CB1 receptor high constitutive activity at both levels of MAPK and adenylil cyclase, in transfected CHO cells (Bouaboula et al., 1997) and exhibits a significant anti-tumor effects in tumor xenografts induced by the sub-cutaneous injection of KiMol cells (Bifulco et al., 2004).

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Furthermore, SR141716 can act as anti-proliferative molecule in pre-adipocyte cells *in vitro* (Gary-Bobo et al., 2006). However, the mechanism by which SR141716 exerts these effects both *in vitro* and *in vivo* remains unknown.

Some observations on the caveolae/lipid rafts-mediated uptake of CB1 ligand anandamide (Mc Farland et al., 2004), the intracellular trafficking and regulation of CB1 receptor-mediated signal transduction (Bari et al., 2005) suggest that there are connections between CB1 activity and cholesterol enriched lipid rafts. Interstingly, we have previously found that CB1 receptor is associated with lipid rafts/caveolae in MDA-MB-231 breast cancer cells (Sarnataro et al., 2005). Furthermore, CB1 plasma membrane distribution and its raft-association are dependent on cholesterol levels and on ligand binding suggesting that the membrane distribution of the receptor is dependent on rafts and is possibly regulated by the agonist binding. Specifically, lipid rafts have been implicated in protein sorting and membrane trafficking in many cell types (Simons and Ikonen, 1997) and may serve as foci for recruitment of signalling molecules at the plasma membrane and thus have been implicated in signal transduction from cell surface receptors (Simons and Toomre, 2000). In addition, it has been reported that lipid rafts/caveolae play a key role in breast tumour cells invasion (Bourguignon et al., 2004). At the light of these findings we reasoned that caveolae/lipid rafts might be involved in CB1-dependent signalling. Thus, the question of whether CB1-mediated signalling plays any role in regulating breast cancer cell growth and to characterize the mechanisms underlying this event is the focus of the present study.

In order to elucidate the mechanism of these transduction pathways that lead to the anti-proliferative CB1-mediated effects, we utilized the selective antagonist SR141716 in MDA-MB-231 human breast cancer cells *in vitro* and in nude mice subcutaneously injected with the same cells. We investigated the role of caveolae/lipid rafts in CB1-mediated MAPK activation

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and we proposed a novel molecular mechanism involved in the anti-proliferative effect induced by the selective CB1 antagonist.

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Materials and Methods

Animals. Adult female nude (CD1) mice were purchased from the Charles River Laboratories s.r.l. The mice were housed in polyethylene cages and given rodent chow and water ad libitum. Mice were housed in rooms maintaining a controlled temperature and on a 12-h light/dark cycle.

Drugs and antibodies. The selective CB1 antagonist, SR141716, was provided by Sanofi-Aventis (Montpellier, France). Anti-ERK1/2 antibodies were purchased from Chemicon (Temecula, Calif). The anti-CB1R antibody was purchased from Santa Cruz Biotechnology and anti-caveolin 1 antibody was purchased from BD Biosciences. MCD was purchased from Sigma (St Louis, Mo).

Cell culture. Human breast cancer cell line MDA-MB-231 (invasive, metastatic, ER-), T47D and MCF-7 (less invasive, ER+), were grown respectively in RPMI 1640 medium or DMEM with 10% foetal bovine serum (FBS) and 2 mM L-glutamine. CHO cells (negative for CB1 receptor expression) were grown in Ham's F12 plus 10% FBS. Human lymphocytes from three healthy donors were separated and cultured in RPMI 10 % FBS, 2 mM L glutamine, and 10 μg/ml phytohemoagglutinin (PHA, M form). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. Cell culture reagents were all obtained from Gibco (Grand Island, NY).

Proliferation assay. The effects of SR141716 on breast cancer cell lines and CHO proliferation were evaluated, *in vitro*, by [^{3}H]thymidine incorporation assay. In brief, $5x10^4$ cells/ml were seeded into 96-well plates and immediately treated with the drug, incubated for 24 h, then pulsed with 0.5 μCi/well of [^{3}H]thymidine and harvested 4 h later. Radioactivity was measured in a scintillation counter (Wallac, Turku, Finland).

Treatment of cells with CB1 receptor sense and antisense oligodeoxynucleotides. Phosphorothioate oligonucleotides were purchased from Primm Srl (Milan, Italy). The antisense

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probe is a 18-mer (5'-GTACTGAATGTCATTTGA-3'), complementary to 73-90 positions of the human CB1 cannabinoid receptor mRNA codon. The corresponding sense probe (5'-TCAAATGACATTCAGTAC-3') was used as a control. MDA-MB-231 cells plated in 6-well were washed 3 times with an Invitrogen pre-warmed Opti-MEM. To each well, 100 μl of Opti-MEM containing 4 μl of lipofectin and oligonucleotides (1 μM final concentration) were added. After incubation for 4 h at 37°C, the medium was replaced with the appropriate cell growth medium containing 1 μM of the same oligonucleotide, and the cells were incubated for an additional 24 h. Following oligonucleotide treatment, cells were stimulated with SR141716 and tested for [^{3}H]-thymidine incorporation.

Chromosome aberrations and mitotic index assay. For cytogenetic analysis, human lymphocytes and CHO cells ($1x10^6$ cells/plate) were cultured for 48 h. SR141716 was added immediately after PHA stimulation (human lymphocytes) or cell adhesion (CHO cells) and left throughout the culture period. Colcemid (Sigma) 0.2 μg/ml was added 2 h before cells were processed and chromosome preparation was performed as previously described. From each treatment, 50 well spread metaphases were scored for the detection of chromosome aberrations (Lioi et al., 1998; Santoro et al., 2005). Mitotic index was determined as the percentage of metaphases over a total of 1000 nuclei analysed at random.

Assessment of apoptosis by annexin-V/propidium iodide double-staining assay. The cells were incubated for 24 h with SR141716, then collected, washed with PBS and resuspended at $1x10^6$ cells/ml in annexin V binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2,5 mM $CaCl_2$). Apoptotic cell death was identified by double supra-vital staining with recombinant FITC-conjugated annexin-V-antibody (Dakocytomation) and propidium iodide. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and

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analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software.

Cell cycle analysis. Cells were collected, fixed in 300 µl of PBS plus 700 µl of ethanol 70% and kept at -20ºC o.n. Propidium iodide (10µg/ml) in PBS containing 100 U/ml DNase-free RNase A was added to the cells; after 15 min at room temperature cells were subjected to flow cytometric analysis using ModFit LT v3.0 from Verity Software House, Inc. (Topsham, ME) program. Each sample was analysed using 10,000 events corrected for debris and aggregate populations.

Western blot analysis. Cell lysates were obtained and processed for western blot analysis as previously described (Sarnataro et al., 2005).

GM1 Determination. A drop of 30 µl of each OptiPREP^{TM} gradient fraction pool was spotted on nitrocellulose membrane, applying vacuum for few seconds. The membrane was saturated for 2 h in 5% milk in PBS/0.1% Tween and then incubated 1 h with Cholera Toxin-HRP (Sigma) and revealed by ECL.

Reverse transcriptase polymerase chain reaction. Total RNA was extracted from cell lines by guanidinum thiocyanate-isopropanol method. Reverse transcription (RT) was performed using Moloney murine leukaemia virus reverse transcriptase and random oligonucleotide primer. The first strand cDNA was then amplified using two different sets of primers. The sense primer CB1-F (5'-GATGTCTTTGGGAAGATGAACAAGC-3') and the antisense primer CB1-R (5'-GACGTGTCTGTGGACACAGACATGG-3') were used to amplify the CB1 receptor; the primers for amplification of alpha actin were A1F (5'-ATGATCTGGACCATCATCCT-3') and A1R (5'-CTRATGTGGAAGTTRTGCATG-3'). Polymerase chain reactions were performed 30 sec at 93°C, 1 min at 59°C and 1 min at 69°C

for 25-28 cycles. Amplified DNA was extracted with chloroform and electrophoresed in a 2% agarose gel in 0,5 x TBE.

Tumor induction in mice. All experiments were performed in 12-week-old female nude (CD1) mice. Tumors were induced by subcutaneous inoculation (day 0) on the right flank of $2x10^{6}$ MDA-MB-231 cells. When tumors had reached a visible volume (15 day later) animals were divided in two groups, control and treated group. SR141716 was dissolved in 0.2 mL of sterile saline solution (0.9% NaCl) and subcutaneously (s.c.) injected at the previous inoculation site (0,7 mg/Kg/dose). Control group was treated with the same volume of saline solution alone. Treatment was repeated at 72 h intervals. Tumor diameters were measured with callipers every other day until the animals were killed. Tumor volumes (V) were calculated by the formula of rotational ellipsoid: V= A x B 2 /2 (A = axial diameter, B = rotational diameter). After 20 days the experiment was stopped, the animals were killed, and the tumor weight was evaluated. During the treatment, none of mice showed signs of wasting or other visible indications of toxicity. Furthermore, the dose of SR141716 used induced no detectable reduction of the spontaneous activity, as we observed unimpaired locomotion of the treated mice. All mice were maintained at the Biology and Pathology Animal Facility Department, and all animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and the Italian regulation for the welfare of animals in experimental neoplasia (Portella et al., 2003).

Statistical analysis. All data were presented as means ± SE. Statistical analysis was performed using one-way ANOVA. In the case of a significant result in the ANOVA, Student T test was used for dose-response curve and Bonferroni's test for post hoc analysis for all other experiments. A *p* value less to 0.05 was considered statistically significant.

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Assays for DRM-association and cholesterol depletion. Where indicated, MCD (10 mM) was added to the cells in serum free medium for 15 min at 37°C to allow cholesterol depletion (~60%). OptiPREP™ gradient analysis of TX-100-insoluble material in normal conditions or after cholesterol depletion was performed using previously published protocols (Sarnataro et al., 2005; Broquet et al., 2003). Briefly, cells were grown to confluence (about 70%) in 100 mm dishes, treated or not with SR141716 0.1 μM or MCD+SR141716 for indicated times, washed in PBS C/M and lysed for 20 min in TNE/TX-100 1% buffer (25mM Tris-HCl [pH 7,5], 150mM NaCl, 5mM EDTA, 1%TX-100) on ice. Lysates were scraped from dishes, brought to 40% OptiPREP™, and then placed at the bottom of a centrifuge tube. A OptiPREP™ gradient (5-35% TNE) was layered on top of the lysates. One ml fractions (12 fractions in total) were harvested from the top of the gradient. Specifically, starting from the top of the gradient the fractions 4 and 5 (representing rafts) and fraction 9-12 (representing non raft-fractions) were pooled separately and named R and NR, respectively. The presence of CB1R was revealed by western blotting using the anti-CB1R antibody.

Cholesterol determination: colorimetric assay. MDA-MB-231 cells were washed twice with PBS, lysed with appropriate lysis buffer and Infinity Cholesterol Reagent (Sigma Chemical Co. ST Louis, Mo, code number 401-25 P) was added to the lysates in the ratio 1:10 for 5 min at 37°C (according to the suggested Sigma protocol number 401). The samples were then measured in a spectrophotometer at 550 nm.

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RESULTS

Effect of SR141716 on tumor cell proliferation. Analysis of cytogenetic effects.

In order to check the effect of SR141716 on tumor cell proliferation *in vitro*, different models such as highly invasive metastatic ER-, MDA-MB-231 breast cancer cells, less invasive ER+ T47D and MCF-7 cells, and CHO cells (which do not express CB1 receptor) were tested for [^{3}H]-thymidine incorporation levels using different doses of SR141716 (Fig. 1A). SR141716 significantly inhibited MDA-MB-231, T47D and MCF-7 cell proliferation in a dose-dependent manner. Furthermore, we check for SR141716 concentrations comprised between 0 and 0,1 μM in MDA-MB-231 cells, and we did not find any remarkable effect on proliferation (data not shown). However, the growth of highly invasive metastatic MDA-MB-231 cells was inhibited more efficaciously than that of less invasive T47D and MCF-7 cells (Fig. 1A). Of note, this effect was completely lacking in the absence of the CB1 receptor (see Fig. 1A, CHO cells) suggesting that the anti-proliferative effect of SR141716 was CB1-dependent. Interestingly, SR141716 0.1 μM induced the highest inhibition of breast cancer MDA-MB-231 cell proliferation thus suggesting that this effect depends on the dose used but it could also dependent on the expression levels of CB1 receptor.

Indeed, in order to investigate whether the SR141716 effect was a consequence of different expression levels of CB1 receptor in the used breast cancer cell lines, we performed both western blots and RT-PCR analysis for the receptor in MDA-MB-231, MCF7 and T47D cells. As shown in figure 1B the CB1 expression levels were higher MDA-MB-231 compared to MCF7 and T47D cells (Fig.1B). As expected we did not detect CB1-receptor mRNA expression in CHO cells. These results suggest that the higher anti-proliferative effect of SR141716 in MDA-MB-231 cells could be related to the higher expression levels of the receptor in this cell line.

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To confirm the involvement of CB1 in anti-proliferative effect induced by SR141716 in MDA-MB-231 cells, we performed an anti-sense knock-down strategy. MDA-MB-231 cells were pre-incubated 24 h with CB1 receptor sense or antisense oligonucleotides. As expected, in cells pre-incubated with sense oligonucleotides SR141716 inhibited proliferation. However, in cells pre-incubated with antisense oligonucleotides SR141716 failed to inhibit proliferation, reflecting a decline of functional CB1 receptors due to the inhibition of mRNA translation (Fig. 1C).

Moreover, at the same concentrations of SR141716 used in the [^{3}H]-thymidine incorporation proliferation assay, we observed a reducing trend of cell proliferating ability also in human lymphocytes, as detected by the mitotic index analysis (Figure 1C) (similar results were obtained with thymidine incorporation, data not shown). On the other hand, either at 0.1 μM or at all doses tested in tumor cells, SR141716 failed to induce any kind of chromosome aberrations (CAs). In order to assess whether in the absence of the CB1 receptor expression, SR141716 could induce a clastogenic effect *per se*, CAs were evaluated also in CHO cells (Fig. 1D), but no clastogenic effects were found, thus suggesting that SR141716 inhibits breast tumor cell proliferation, being more effective in MDA-MB-231 cells, without inducing a cytogenetic damage nor exhibiting a genotoxic activity in normal cells.

Effect of SR141716 on cell cycle progression.

Since the inhibitory effect of SR141716 on MDA-MB-231 cell proliferation was higher than the one observed in less invasive and non-metastatic cell lines, we further investigated the effects of SR141716 on this more invasive cell line, using 0.1 μM dose. The anti-proliferative effect of SR141716 was not accompanied by apoptosis or necrosis as revealed by a flow cytometric assay with annexinV/propidium iodide double staining (Fig. 2A). To determine how SR141716 influences cell growth, cell cycle phase distribution of asynchronous cells was

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assessed by flow cytometry in MDA-MB-231 cells treated with SR141716 (0.1 μM) for 24 h. In asynchronous, rapidly proliferating cell populations, 36,9%, 39,7%, and 23,4% of cells were found in G0/G1, S, and G2/M phases of the cell cycle, respectively (Fig. 2B, panel a). We found that SR141716 induced a reduction in the percentage of cells in S phase (28,8%) with a parallel increase of cells in G0/G1 (46,6%) (Fig. 2B, panel a). This effect was also observed when cells were pre-synchronized by serum starvation. In this case we found that SR141716 induced a strong increase in the percentage of cells in G0/G1 (44,3%) compared to the control (27,5%, Fig. 2B, panel b).

These results confirm the inhibition of proliferation at the G1 phase of the cell cycle. Therefore, to determine whether these changes in the cell cycle progression were related to different expression of cell cycle regulatory proteins implicated in the control of G1/S transition, western blotting of cell lysates were carried out using immunodetection of various cell cycle-related proteins. Among these, cyclin D1 and E were reduced by SR141716 after 24 hr treatment (Fig. 2C). In addition, as expected, the expression of cyclin-dependent kinases (cdk) inhibitors $p27^{KIP1}$, which is known to mediate cell cycle arrest in response to various anti-proliferative signals was increased by SR141716 treatment (Fig. 2C).

SR141716 reduced the tumor volume in vivo in xenograft tumors.

To evaluate *in vivo* the efficacy of SR141716 treatment on MDA-MB-231 cell growth, nude mice were injected s.c. with $2x10^6$ MDA-MB-231 cells. When tumors were clearly detectable (15 days following injection), the mice were exposed to either vehicle or SR141716 (0,7mg/Kg/dose). Tumor growth was monitored and the data revealed that exposure to SR141716 led to a significant decrease in tumor mass compared to vehicle control-treated mice (Fig. 3). Animals exhibited no outward signs of toxic or hypolocomotor effects, nor effects of

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SR141716 (0,7mg/kg/dose) on animal weight gain, haematocrit or WBC levels have been revealed (data not shown).

Mechanism underlying SR141716 anti-proliferative effect.

We have recently shown that the CB1 cannabinoid receptor is associated with lipid rafts/caveolae in MDA- MB-231 cells (Sarnataro et al., 2005). It has been proposed that the function of lipid rafts/caveolae is the spatial concentration of specific sets of protein in order to increase the efficiency and specificity of signal transduction by facilitating interactions between proteins and by preventing cross-talk between pathways (Moffett et al., 2000). Moreover, as endocannabinoids through CB1R-dependent mechanism have been shown to have a role in cancer (De Petrocellis et al., 1998; Grimaldi et al., 2006; Di Marzo et al., 2004) and lipid rafts play a pivotal role in breast tumor cell invasion (Bourguignon et al., 2004), we decided first to check whether the CB1 receptor associated with lipid rafts in the presence of its antagonist SR141716 and subsequently to investigate the role of raft microdomains in SR141716 signalling by using a cholesterol sequestering agent methyl-β-cyclodextrin (MCD), which is known to perturb lipid rafts composition (Sarnataro et al., 2002; Paladino et al., 2004).

Having previously documented and here reconfirmed (Fig. 4, panel a) the enrichment of CB1R in lipid rafts/caveolae, we next examined the subcellular distribution of a signalling molecules relevant for CB1R signal transduction, ERK 1 and 2 and its phosphorylated form (pERK). As indicated in the Fig. 4A (panel a), in control steady-state conditions (24 hr) about 20% of total ERK was present in the raft fraction (R). Interestingly, in starved MDA-MB-231 cells stimulated for 15 min with 10% serum, only 20% of CB1R and 3-5% of total ERK 1/ 2 were present in lipid rafts while**,** as predicted pERK 1/ 2 were totally absent from them. These data suggests that after serum starvation and subsequent 15 min serum incubation the composition

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of lipid rafts is perturbed, not completely restored and consequently the association of the protein within is slightly affected compared to the control conditions (Fig. 4A, compare 24hr and 15 min, control).

In order to directly test the influence of SR141716 on the association of CB1R with lipid rafts, we included SR141716 (0.1 μM, 24 hr) in the culture medium of MDA-MB-231 cells and then cell lysates were subjected to OptiPREP^{TM} density gradient flotation assay in TX-100 detergent. Pooled fractions 4-5 (representing the raft fractions, R) and 9-12 (representing non raft fractions, NR) were resolved by SDS-PAGE and immunoblotted to determine the relative abundance of proteins within. Incubation with SR141716 for 24 hr (Fig. 4A), almost completely abolished the receptor raft-association, inducing CB1R to be excluded from lipid rafts thus redistributing in the heavy fractions of the gradients (from the raft fractions, R to the non-raft fractions, NR). The presence of a typical rafts marker caveolin-1 (cav-1) (Sarnataro et al., 2005; Bourguignon et al., 2004) and of an extensively characterized raft marker GM1 (Sarnataro et al., 2004; Paladino et al., 2004; Bender et al., 2003) in the 5-30% OptiPREP gradient interface (lipid rafts, R), indicates that we had successfully isolated rafts/caveolae microdomains. We also verified that a typical non-raft marker, the endoplasmic reticulum resident protein Bip/GRP78, was excluded from these fractions. Of note, the described effect of SR141716 was specific for the CB1 receptor, as caveolin-1 and the other molecules regularly distributed in the lipid rafts fraction both in control and SR141716 treated samples (see Fig. 4 at 24hr, panels a and b). In light of these data, in order to understand whether the described effects of SR141716 might have some implications at molecular signalling levels, we verified the expression levels of ERK1/ 2 MAPK phosphorylated isoforms by checking with immunoblot analysis for pERK 1/ 2, and for the presence of CB1R in rafts fraction after 15 min incubation with SR141716. As shown in the Fig. 4 (panel b) about 50 % of CB1R enriched lipid rafts and,

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as well as in control condition and previously demonstrated in another cell type (Yang et al., 2004), 20% of total ERK 1/ 2 were present in detergent resistant domains (DRMs or rafts). Intriguingly, treatment with SR141716 for 15 min lowered the expression of pERK1/ 2 compared to the control conditions.

To directly evaluate the role of lipid raft integrity in the functional effects of SR141716, we first perturbed MDA-MB-231 rafts composition with methyl-β-cyclodextrin - which we have shown to displace the CB1 receptor from lipid rafts (Sarnataro et al., 2005) - and subsequently checked for the gradient distribution of the CB1 receptor in the OptiPREP preparation after cellular incubation with SR141716 (0.1 μM). As shown in the Fig. 4 (MCD+SR141716, panel c), CB1R remained in the heavy fractions of the gradients (NR) as well as total ERK 1/ 2 and pERK. Of note, under these conditions pERK protein levels were comparable to the ones of control conditions. Therefore, the inhibitory effect of SR141716 on ERK 1/ 2 activation in MDA-MB-231 cells was reverted after pre-treatment with MCD (Fig. 4, panel c, 15 min) indicating that SR141716 needs rafts integrity to function.

To gain further insights into the involvement of lipid rafts in the anti-proliferative effect induced by SR141716, MDA-MB-231 cell growth was tested following treatment with SR141716 (0.1 μM) in basal conditions and after perturbation of lipid rafts composition with MCD (10 mM). Both cell count and [^{3}H]-thymidine uptake into cells were used and a close correlation between these two parameters was observed. Interestingly, we found that the anti-proliferative effect exhibited by SR141716 was almost completely reverted by pre-treatment with MCD (Fig. 5, MCD+SR141716) indicating again that the cell growth arrest induced by the CB1 antagonist needs integrity of lipid rafts to occur. In support of our data, there is a large body of evidence that G protein-coupled receptors functions depend on the lipid rafts integrity (Simons and Toomre, 2000; Pike et al., 2003; Simons and Ehehalt, 2002). Of note, as

previously described in another cell line (Choi et al 2004) MCD alone induced per se a little anti-proliferative effect.

Thus, to our surprise, CB1R antagonist SR141716, although redistributes the receptor in the "non-raft" fractions, displays a significant anti-proliferative effect on MDA-MB-231 cells (see Fig. 5) and cholesterol depletion by MCD totally abolishes the SR141716-induced cell growth arrest. Therefore, it is conceivable that SR141716 needs the "raft integrity" to exert its anti-proliferative property through CB1R-signalling.

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DISCUSSION

The present findings provide unequivocal evidence for the role of the CB1 receptor and its antagonist/inverse-agonist rimonabant (SR141716) in breast tumor cell proliferation. Our data also indicate that the molecular mechanism at the basis of SR141716 function, linked to CB1 receptor, needs lipid rafts/caveolae integrity to occur.

The endocannabinoid system is an almost ubiquitous signalling system involved in the control of cell fate. Recent studies have investigated the possibility that drugs targeting the endocannabinoid system might be used to retard or block cancer growth. The endocannabinoids have been shown to inhibit the growth of tumor cells in culture and animal models by modulating key cell-signalling pathways (Guzman, 2003; Bifulco and Di Marzo, 2002). We have also previously reported that the endocannabinoid anandamide inhibits K-*ras* oncogene-dependent tumor growth through the CB1 cannabinoid receptors, thus indicating that endocannabinoid-based drugs may be efficacious therapeutic drugs for the inhibition of cancer cells growth (Portella et al., 2003; Bifulco et al., 2001).

Since SR141716 functions as a specific CB1 receptor antagonist, it could be expected that it would induce, if used alone, null or opposite effects to the anti-proliferative activity exerted by CB1 agonist molecules (*e.g.* anandamide). The absence of proliferation stimulation by SR141716 is, indeed, not surprising as it indicates that, although endocannabinoids might help down-regulating tumor growth *in vivo*, this represents only one out of many possible tumor-suppressing mechanisms occurring during cell transformation.

Indeed we found that SR141716 has a significant and dose-dependent anti-proliferative property, through the CB1 receptor, in breast cancer cells such as T47D, MCF-7 and MDA-MB-231 cells. However, the inhibition of cell proliferation seems to depend also on the expression levels of the receptor in the different breast cancer cells used and on the less or more invasive

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phenotype of the breast cancer cell lines. Although SR141716 could exert the observed anti-proliferative effect in MDA-MB-231 cells via a non-CB1 receptor dependent mechanism, results with antisense oligonucleotides provide more definitive evidence for the involvement of CB1 receptor.

We observed that the highest inhibition of cell proliferation was reached in MDA-MB-231 at 0,1 μM, without inducing apoptosis and/or necrosis. Of note, this concentration did not exert any cytotoxic effect in human lymphocytes. Since most drugs, especially chemotherapeutics, induce adverse health effects related to their genetic toxicological activity (genotoxicity), to test the genotoxic potential of SR141716 we used a sensitive and rapid procedure such as chromosome aberration analysis in human lymphocytes and CHO cells (Puck et al., 2002). We did not observe any kind of chromosome aberrations on normal cells, human lymphocytes and in CHO cells, even in the concentration range at which we could observe the above anti-proliferative effect in breast cancer cells. It is important to underline that the lack of genotoxicity suggests that SR141716 is not carcinogenic and gives useful information for both follow-up evaluation and its potential clinical use (Dearfield et al., 2005).

In order to gain further insight into the anti-proliferative effect, we analysed the cell cycle progression in SR141716-treated MDA-MB-231 cells. We observed that SR141716 is able to exert an anti-proliferative effect by blocking the G0/G1 transition, similarly to what we had previously observed with endocannabinoids (*e.g.* anandamide) in MCF-7 cell line (De Petrocellis et al., 1998). This effect was also confirmed by both the inhibition of cyclin E and D1 expression levels and the increase of the cdk inhibitor $p27^{KIP1}$.

On the other hand, we also found that SR141716 inhibits MDA-MB-231-induced tumor growth *in vivo*, the effect being statistically significant starting from at least three weeks of treatment; the overall observations suggest an anti-tumor activity of SR141716.

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Furthermore, we studied in details the molecular mechanism underlying SR141716 anti-proliferative effect. Methyl-β-cyclodextrin, a cholesterol sequestering agent which is known to perturb lipid rafts composition has been described also to have a cell growth inhibition effect in macrophage cells (Choi et al., 2004). Interestingly, MCD alone exhibits a small anti-proliferative effect also in MDA-MB-231 cells and cholesterol depletion by MCD treatment of cells before SR141716 incubation induces CB1R to be excluded from lipid rafts (Fig. 4) and causes a reversion of anti-proliferative effect of SR141716 (Fig. 5). This observation is particularly intriguing because endocannabinoids through CB1R-dependent mechanism have been shown to have a role in cancer (Guzman, 2003; Bifulco and Di Marzo, 2002) and lipid rafts play a critical role in breast tumor cell invasion (Bourguignon et al., 2004). Complementarily with these data, we have previously shown that CB1R is associated with lipid rafts/caveolae and suggest that they might represent a cellular device for its intracellular trafficking as well as favourable platform to regulate CB1R signalling (Mc Farland et al., 2004; Bari et al., 2005). Given these informations, we can speculate that the clear, albeit small anti-proliferative effect of MCD is likely associated with its affinity for cell membrane components, particularly cholesterol, which plays a major role in the structure and function of the cell membrane (Jadot et al., 2001, Steck et al., 2002). Moreover MCD is known to interact with the lipid components of biological membranes and to modulate their fluidities and permeabilities (Hartel et al., 1998).

In this study we have checked the implication of lipid rafts/caveolae in the signalling deriving from SR141716 through CB1 receptor interaction. As previously shown by Bouaboula and colleagues (1997), SR141716 inhibits MAPK activity only in cells over-expressing CB1 receptor. Interestingly we found that SR141716 decreases ERK1/2 activity and cholesterol depletion by MCD pre-treatment reverts SR141716 inhibitory effects on ERK1/2 in MDA-MB-

231 cells (Fig. 4), indicating again that SR141716 signalling requires lipid rafts integrity to occur and that the role of lipid rafts in the receptor-dependent signalling would be to render favourable the CB1R-ligand (SR141716) encounter on the cell surface, and/or at least the formation of the molecular machinery responsible for CB1-dependent signalling.

Furthermore, our data support the view that perturbation of lipid rafts/caveolae might represent a useful tool to control CB1R signalling and could provide new insights towards a better understanding of endocannabinoid-signalling regulated malignancy of human breast cancer.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1: Cell proliferation and cytogenetic effects of SR141716. A) MDA-MB-231, T47D, MCF-7 and CHO cells ($5x10^4$/well) were cultured in triplicate for 24h with concentrations of SR141716 ranging from 0.1 to 1.0 μM. After 24 h incubation, [^{3}H]-thymidine incorporation (0.5 μCi/well) was measured. The graphs report the mean of c.p.m. ± SE values of three independent experiments. Results were statistically significant (ANOVA, $p<0.01$ for MDA-MB-231, T47D and MCF-7 cells, with Student's t test *vs* control, * $p<0.05$, ** $p<0.01$). B) Comparation of CB1R expression levels in the three breast cancer cell lines. Panel a) cell lysates (50 μg total proteins) were subjected to SDS-PAGE. CB1R was revealed by western blotting on nitrocellulose and hybridization with polyclonal anti-actin antibody as loading control. The experiment was repeated three times. Panel b) for RT-PCR the cells (MDA-MB-231, MCF-7, T47D and CHO) were subjected to total RNA extraction and reverse transcription (RT) was performed using Moloney murine leukaemia virus reverse transcriptase and random oligonucleotide primers for CB1R and actin were used. Polymerase chain reactions were performed 30 s at 93 °C, 1 min at 59 °C and 1 min at 69 °C for 25–28 cycles to assess saturation of the signal. C) Cell proliferation in MDA-MB-231 cells trasfected with CB1 receptor sense and antisense oligonucleotides as described in Materials and Methods. MDA-MB-231 cells ($5x10^4$/well) were cultured in triplicate for 24h with 0.1 μM of SR141716. After 24 h incubation, [^{3}H]-thymidine incorporation (0.5 μCi/well) was measured. The graphs report the mean of c.p.m. ± SE values of three independent experiments. Results were statistically significant (ANOVA, $p<0.01$, with Student's t test SR141716 (sense ODN) *vs* control, ** $p<0.01$ and SR141716 (antisense ODN) *vs* SR141716 (sense ODN), * $p<0.05$). D) SR141716 is not genotoxic in normal cells. Chromosome Aberrations (CAs) and Mitotic index (MI) (determined as indicated in Materials and Methods) were evaluated in human

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lymphocytes (panel a) and in CHO cells (panel b) treated for 48 h with increasing concentrations of SR141716. The graphs report the mean ± SE values of three independent experiments. Results were statistically significant (ANOVA, $p<0.01$, with Student's t test *vs* control, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Fig. 2: Effect of SR141716 on apoptosis and cell cycle progression of MDA-MB-231 cells. A) MDA-MB-231 cells were incubated for 24 h with SR141716 0.1 μM; then cells were processed and apoptotic cell death was analysed as described in Methods. The panels relative to control (a) and SR141716 treatment (b) are representative of three independent experiments. As positive control of the procedure in the panel (c) the capacity of pyrrolidine dithiocarbamate (PDTC) to induce apoptosis in MDA-MB-231 cells is shown (see also Daniel et al., 2005 and Supplemental Data Fig. 1S). B) SR141716 alters cell cycle progression. MDA-MB-231 treated with SR141716 at 0.1μM for 24 h were stained with propidium iodide and analysed by flow cytometry. Data were subjected to ModFit analysis. Control and SR141716 panels show the relative percentage of cells in the G0/G1, S and G2/M phases and the SE of the combined results of three independent experiments (indicated in parenthesis). The panels are relative to asynchronous (a) and serum starved cells (b). C) The effect of SR141716 on the expression levels of cyclin D1, E and p27 ^{KIP1} was evaluated by western blot analysis on cell lysates. In brief, MDA-MB-231 cells grown under control or SR141716 0.1 μM conditions were lysed as described in materials and methods (in the section western blot) and an equal amount of total proteins (50 μg) was loaded on gel, resolved by SDS-PAGE and western blotted with antibodies against the specific proteins. The same filters were stripped and reprobed with rabbit polyclonal anti-actin antibody as loading control. Immunoreactive bands,

from three independent experiments were quantified using Quantity One program and normalized to actin bands.

Fig. 3: SR141716 inhibits growth of xenograft tumors induced by MDA-MB-231 injection in mice. Tumor volume at different weeks from mice inoculation. Data were shown as mean ± SE of n=5 animals. Differences in tumor volumes after 3 weeks were significant (p<0,05 by ANOVA followed by Bonferroni's test, *** p<0.001 for SR141716-treated group *vs* control group at three weeks).

Fig. 4: Effect of SR141716 on CB1R raft-association after lipid rafts perturbation. MDA-MB-231 cells were incubated with indicated drugs for 24 hr or for 15 min after serum starvation and lysed for 20 min in cold TNE/TX-100 buffer. 500 µg of total proteins were run through a "Two Step" OptiPREP^{TM} gradient (5-40% OptiPREP). One-milliliter fractions were collected from the top of the gradient after centrifugation to equilibrium. Both lipid rafts fractions (pooled fractions 4 and 5, R) and the non-raft material (pooled fractions 9-12, NR) were loaded on 12 % gel and revealed by western blotting. Both at 24 hr and 15 min serum incubation after starvation, the distribution of CB1 receptor, of the rafts marker caveolin 1 and of the non-raft protein Bip were analysed by western blot with the specific antibody and ECL. The distribution of GM1 was assessed, on an aliquot of each fraction pool with dot blot analysis using Cholera Toxin-HRP. The levels of total and phosphorylated ERK1/ 2 in R and NR fractions were analysed by stripping nitrocellulose membrane and immunoblotting with the specific antibodies. Data were quantified by densitometric analysis with QuantityOne program. The relative percentage of CB1R, cav-1 and ERK1/ 2 protein content in R and NR were

expressed as percentage of total protein amount in each gradient fraction representing the 100%.

Fig. 5: Evaluation of MAPK activity after lipid rafts perturbation. MDA-MB-231 cells were cultured in triplicate, treated with MCD for 15 min (alone or subsequently with SR141716 for 24 hr) or for 24h with SR141716 0.1 μM. After 24 h incubation, [^{3}H]-thymidine incorporation (0.5 μCi/well) was measured. The graph reports the mean ± SE values of three independent experiments. Results were statistically significant (ANOVA, $p<0.001$, with post hoc Bonferroni test *$p<0.01$ for MCD *vs* control; *** $p<0.001$ for SR141716 *vs* control and for SR141716 + MCD *vs* SR141716).

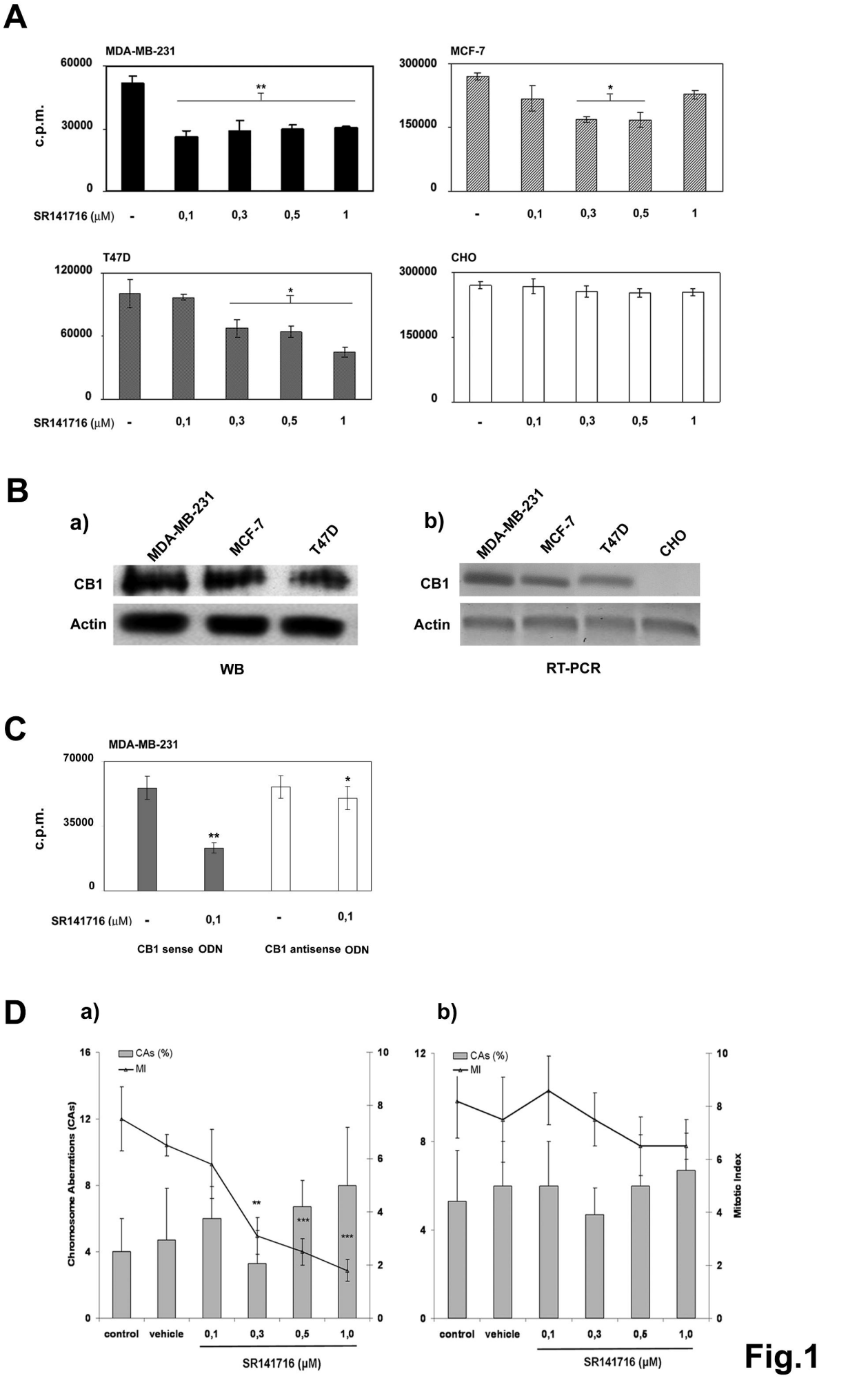
A
MDA-MB-231
MCF-7
T47D
CHO
c.p.m.
SR141716 (μM)
B
a)
b)
CB1
Actin
WB
RT-PCR
C
CB1 sense ODN
CB1 antisense ODN
D
CAs (%)
MI
Chromosome Aberrations (CAs)
Mitotic Index
control
vehicle
SR141716 (μM)

Fig.1

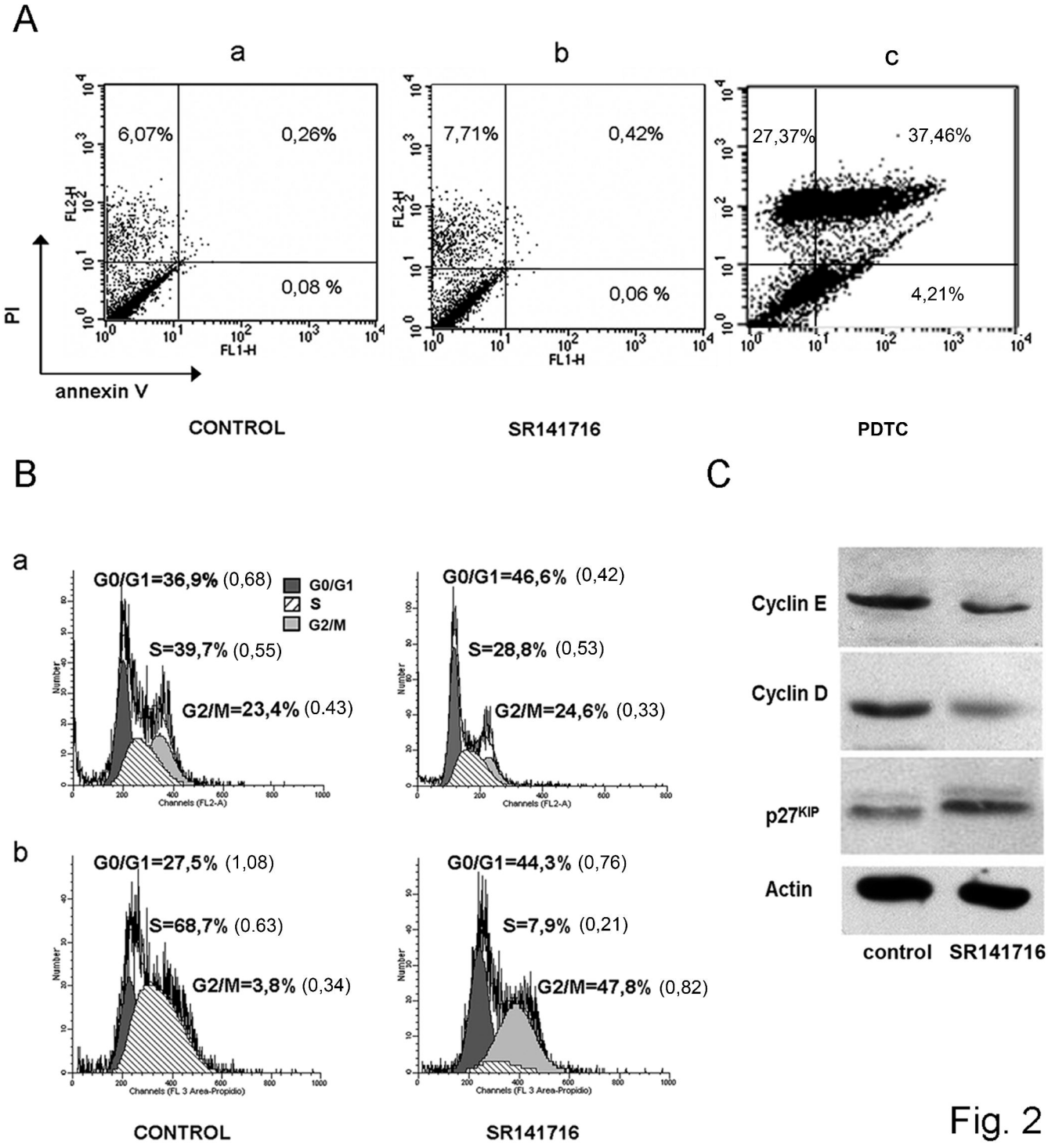
A
a
b
c
6,07%
0,26%
0,08 %
7,71%
0,42%
0,06 %
27,37%
37,46%
4,21%
FL2-H
FL1-H
PI
annexin V
CONTROL
SR141716
PDTC
B
a
G0/G1=36,9% (0,68)
S=39,7% (0,55)
G2/M=23,4% (0.43)
G0/G1
S
G2/M
G0/G1=46,6% (0,42)
S=28,8% (0,53)
G2/M=24,6% (0,33)
Number
Channels (FL2-A)
b
G0/G1=27,5% (1,08)
S=68,7% (0.63)
G2/M=3,8% (0,34)
G0/G1=44,3% (0,76)
S=7,9% (0,21)
G2/M=47,8% (0,82)
Channels (FL 3 Area-Propidio)
CONTROL
SR141716
C
Cyclin E
Cyclin D
p27KIP
Actin
control
SR141716

Fig. 2

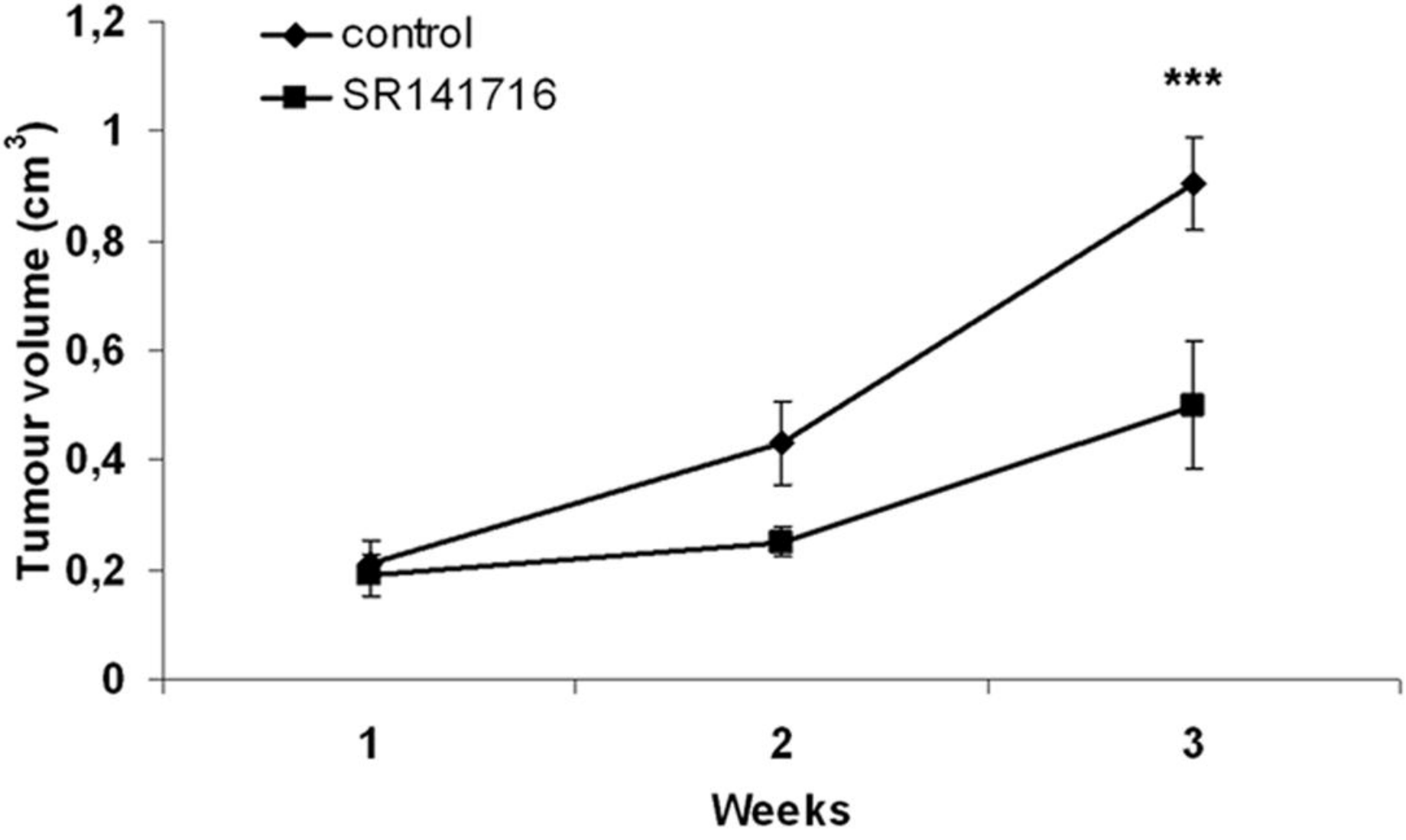
control
SR141716
***
Tumour volume (cm3)
1,2
1
0,8
0,6
0,4
0,2
0
1
2
3
Weeks

Fig. 3

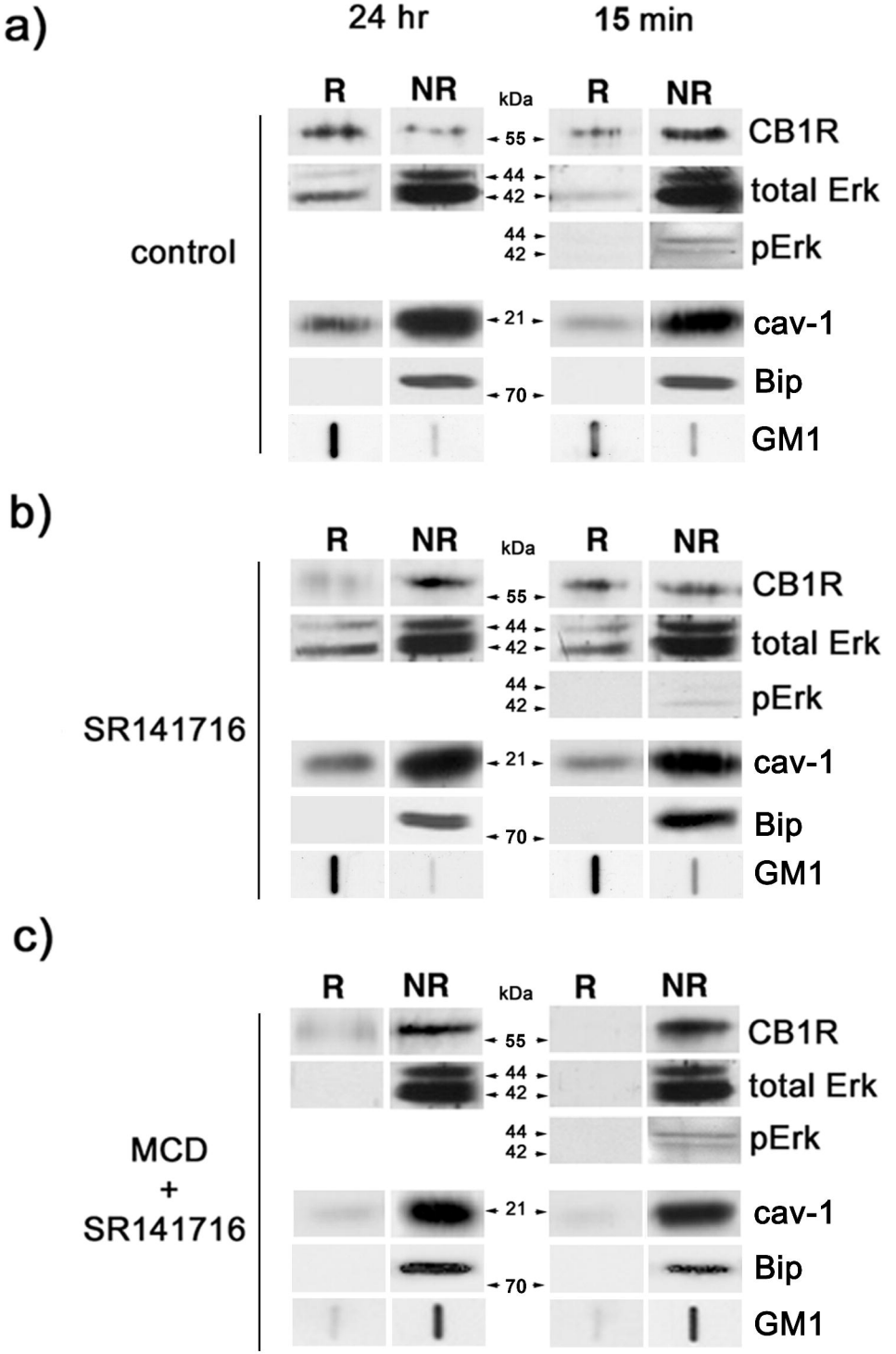
a)
24 hr
15 min
R
NR
kDa
R
NR
CB1R
total Erk
pErk
cav-1
Bip
GM1
control
55
44
42
21
70
b)
SR141716
c)
MCD
+
SR141716

Fig.4

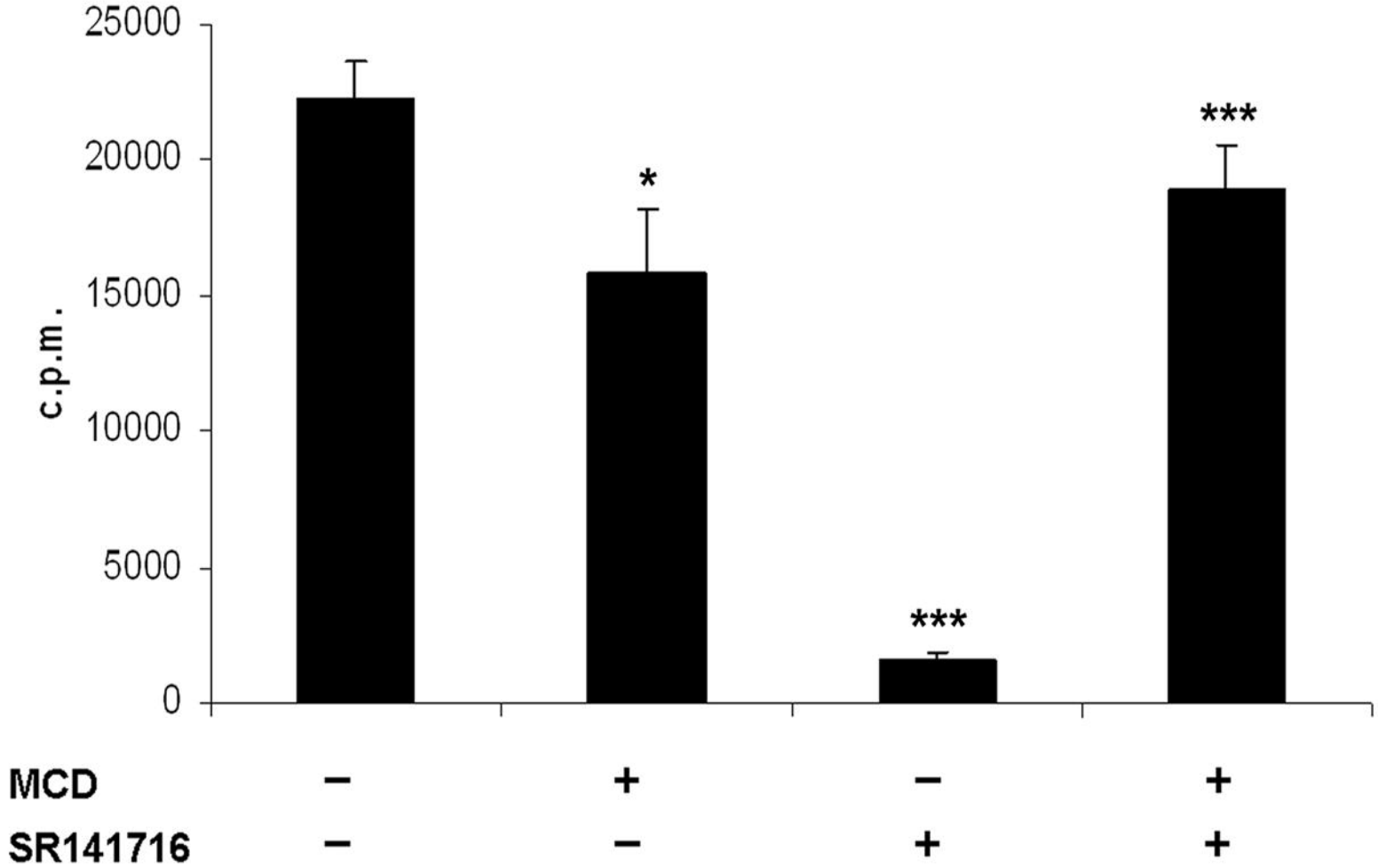

Fig.5