0026-895X/06/7004-1298–1306$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 70:1298–1306, 2006

Vol. 70, No. 4
25601/3139171
Printed in U.S.A.

The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Human Breast Cancer Cell Proliferation through a Lipid Raft-Mediated Mechanism[S]

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Received April 11, 2006; accepted July 5, 2006

ABSTRACT

The endocannabinoid system has been shown to modulate key cell-signaling pathways involved in cancer cell growth. In this study, we show that cannabinoid receptor type 1 (CB1) 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 antiproliferative effect was not accompanied by apoptosis or necrosis and was characterized by a G_1/S-phase cell cycle arrest, decreased expression of cyclin D and E, and increased levels of cyclin-dependent kinase inhibitor $p27^{KIP1}$. We have also shown that SR141716 exerted a significant antiproliferative 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 antiproliferative effect in tumor cells, we did not observe evidence of any genotoxic effect on normal cells. Our data also indicate that the SR141716 antiproliferative effect requires lipid raft/caveolae integrity to occur. Indeed, we found that CB1 receptor (CB1R) is completely displaced from lipid rafts in SR141716-treated MDA-MB-231 cells, and cholesterol depletion by methyl-β-cyclodextrin strongly prevented SR141716-mediated antiproliferative effect. Taken together, our results suggest that SR141716 inhibits human breast cancer cell growth via a CB1R lipid raft/caveolae-mediated mechanism.

The "endocannabinoid system," comprising the cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) receptors, their endogenous ligands (endocannabinoids), and the proteins that regulate endocannabinoid biosynthesis and degradation, controls several biological functions, including food intake in animals (Bensaid et al., 2003; Jbilo et al., 2005) 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 antitumor effects (Guzman, 2003). We have previously observed that endocannabinoids inhibit the proliferation of human breast cancer cells by blocking the G_0/G_1-S-phase transition of the cell cycle through interference with cannabinoid CB1 receptor (CB1R)-coupled signal-transducing events (De Petrocellis et al., 1998).

It seems 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 CB1R 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 described previously that a selective CB1R antagonist, SR141716, but not the CB_2 receptor antagonist, counteracts most of the antitumor effects of anandamide (AEA), suggesting that CB1R are uniquely involved in the effects of this compound (Bifulco et al., 2004; Grimaldi et al.,

This study was supported by Sanofi-aventis (M.B.) and the Associazione Educazione e Ricerca Medica Salernitana, ERMES, as well as by a fellowship from Associazione Italiana Ricerca sul Cancro (A.S.).

D.S. and S.P. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.025601.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; CB1R, CB1 receptor(s); AEA, anandamide; MAPK, mitogen-activated protein kinase; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; MCD, methyl-β-cyclodextrin; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT, reverse transcription; PCR, polymerase chain reaction(s); ANOVA, analysis of variance; CA, chromosome aberration(s); cav-1, caveolin-1; s.c., subcutaneous.

2006). Interestingly, besides its antagonist properties, SR141716 also has inverse agonist effects (Rinaldi-Carmona et al., 1994; Hurst et al., 2002) because it can block CB1R high constitutive activity at both levels of mitogen-activated protein kinase (MAPK) and adenylyl cyclase in transfected Chinese hamster ovary (CHO) cells (Bouaboula et al., 1997) and exhibits a significant antitumor effect in tumor xenografts induced by the s.c. injection of KiMol cells (Bifulco et al., 2004). Furthermore, SR141716 can act as antiproliferative molecule in preadipocyte 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 raft-mediated uptake of CB1 ligand AEA (McFarland et al., 2004) and the intracellular trafficking and regulation of CB1R-mediated signal transduction (Bari et al., 2005) suggest that there are connections between CB1 activity and cholesterol-enriched lipid rafts. Interestingly, we have previously found that CB1R 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 signaling 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 tumor cell invasion (Bourguignon et al., 2004). In light of these findings, we reasoned that caveolae/lipid rafts might be involved in CB1-dependent signaling. Thus, the question of whether CB1-mediated signaling plays any role in regulating breast cancer cell growth, as well as to characterize the mechanisms underlying this event, is the focus of the present study.

To elucidate the mechanism of these transduction pathways that lead to the antiproliferative CB1-mediated effects, we used the selective antagonist SR141716 in MDA-MB-231 human breast cancer cells in vitro and in nude mice s.c. injected with the same cells. We investigated the role of caveolae/lipid rafts in CB1-mediated MAPK activation, and we proposed a novel molecular mechanism involved in the antiproliferative effect induced by the selective CB1 antagonist.

Materials and Methods

Animals. Adult female nude (CD1) mice were purchased from the Charles River Laboratories, Inc. (Wilmington, MA). The mice were housed in polyethylene cages and given rodent chow and water ad libitum. Mice were housed in rooms maintained with 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). Antiextracellular signal-regulated kinase (ERK) 1/2 antibodies were purchased from Chemicon (Temecula, CA). The anti-CB1R antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-caveolin-1 antibody was purchased from BD Biosciences (San Jose, CA). Methyl-β-cyclodextrin (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 in RPMI 1640 medium or Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, respectively. CHO cells (negative for CB1R expression) were grown in Ham's F12 plus 10% FBS. Human lymphocytes from three healthy donors were separated and cultured in RPMI 1640 medium, 10% FBS, 2 mM L-glutamine, and 10 μg/ml phytohemagglutinin (M form). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. Cell culture reagents were all obtained from Invitrogen (Carlsbad, CA).

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, 5×10^4 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 CB1R Sense and Antisense Oligodeoxynucleotides. Phosphorothioate oligonucleotides were purchased from Primm Srl (Milan, Italy). The antisense probe is an 18-mer (5′-GTACTGAATGTCATTTGA-3′), complementary to 73 to 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 six-well plates were washed three times with Invitrogen prewarmed Opti-MEM. To each well, 100 μl of Opti-MEM containing 4 μl of lipofectin and oligonucleotides (1 μM final concentration) was 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. After 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 (1×10^6 cells/plate) were cultured for 48 h. SR141716 was added immediately after phytohemagglutinin 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 described previously. 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 for a total of 1000 nuclei analyzed 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 phosphate-buffered saline (PBS), and resuspended at 1×10^6 cells/ml in annexin V binding buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl, and 2.5 mM $CaCl_2$). Apoptotic cell death was identified by double supravital staining with recombinant fluorescein isothiocyanate-conjugated annexin V antibody (Dako Denmark A/S, Glostrup, Denmark) and propidium iodide. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ) .

Cell Cycle Analysis. Cells were collected, fixed in 300 μl of PBS plus 700 μl of ethanol 70% and kept at −20°C overnight. Propidium iodide (10 μg/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 version 3.0 from Verity Software House, Inc. (Topsham, ME). Each sample was analyzed using 10,000 events corrected for debris and aggregate populations.

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

GM1 Determination. One drop of 30 μl of each OptiPREP gradient fraction pool was spotted on nitrocellulose membrane, with

vacuum applied for a few seconds. The membrane was saturated for 2 h in 5% milk in PBS/0.1% Tween 20 and then incubated 1 h with Cholera Toxin-HRP (Sigma) and revealed by enhanced chemiluminescence.

Reverse-Transcriptase Polymerase Chain Reaction. Total RNA was extracted from cell lines by guanidinium thiocyanate-isopropanol method. Reverse transcription (RT) was performed using Moloney murine leukemia 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 CB1R; the primers for amplification of alpha actin were A1F (5′-ATGATCTGGACCATCATCCT-3′) and A1R (5′-CTRATGT-GGAAGTTRTGCATG-3′). Polymerase chain reactions (PCR) were performed 30 s at 93°C, 1 min at 59°C, and 1 min at 69°C for 25 to 28 cycles. Amplified DNA was extracted with chloroform and electrophoresed in a 2% agarose gel in 0.5× Tris-borate/EDTA.

Tumor Induction in Mice. All of the experiments were performed in 12-week-old female nude (CD1) mice. Tumors were induced by s.c. inoculation (day 0) on the right flank of 2×10^6 MDA-MB-231 cells. When tumors had reached a visible volume (15 days 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 s.c. injected at the previous inoculation site (0.7 mg/kg/dose). The control group was treated with the same volume of saline solution alone. Treatment was repeated at 72-h intervals. Tumor diameters were measured with calipers every other day until the animals were killed. Tumor volumes (V) were calculated by the formula of rotational ellipsoid: $V = A \times B\ 2/2$ (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 of the mice were maintained at the Biology and Pathology Animal Facility Department, and all of the animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health and the Italian regulation for the welfare of animals in experimental neoplasia (Portella et al., 2003).

Statistical Analysis. All of the data were presented as mean ± S.E. Statistical analysis was performed using one-way analysis of variance (ANOVA). In the case of a significant result in the ANOVA, Student's t test was used for dose-response curve, and Bonferroni's test was used for post hoc analysis for all of the other experiments. A p value less than 0.05 was considered statistically significant.

Assays for Detergent-Resistant Domain Association and Cholesterol Depletion. When 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 Triton X-100-insoluble material in normal conditions or after cholesterol depletion was performed using previously published protocols (Broquet et al., 2003; Sarnataro et al., 2005). In brief, cells were grown to confluence (approximately 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 buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100 on ice. Lysates were scraped from dishes, brought to 40% OptiPREP, and then placed at the bottom of a centrifuge tube. An OptiPREP gradient (5–35% TNE) was layered on top of the lysates. One-milliliter 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 fractions 9 through 12 (representing nonraft 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 and lysed with appropriate lysis buffer, and Infinity Cholesterol Reagent (Sigma) was added to the lysates in the ratio 1:10 for 5 min at 37°C (according to the manufacturer's instructions). The samples were then measured in a spectrophotometer at 550 nm.

Results

Effect of SR141716 on Tumor Cell Proliferation: Analysis of Cytogenetic Effects. 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 CB1R) 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 checked 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). It is noteworthy that this effect was completely lacking in the absence of the CB1R (see Fig. 1A, CHO cells), suggesting that the antiproliferative 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 depend on the expression levels of CB1R.

Indeed, to investigate whether the SR141716 effect was a consequence of different expression levels of CB1R 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 Fig. 1B, the CB1 expression levels were higher in MDA-MB-231 compared with MCF7 and T47D cells. As expected, we did not detect CB1R mRNA expression in CHO cells. These results suggest that the higher antiproliferative effect of SR141716 in MDA-MB-231 cells could be related to the higher expression levels of the receptor in this cell line.

To confirm the involvement of CB1 in the antiproliferative effect induced by SR141716 in MDA-MB-231 cells, we performed an antisense knockdown strategy. MDA-MB-231 cells were preincubated 24 h with CB1R sense or antisense oligonucleotides. As expected, in cells preincubated with sense oligonucleotides, SR141716 inhibited proliferation. However, in cells preincubated with antisense oligonucleotides, SR141716 failed to inhibit proliferation, reflecting a decline of functional CB1R caused by 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 (Fig. 1C) (similar results were obtained with thymidine incorporation, data not shown). On the other hand, either at 0.1 μM or at all of the doses tested in tumor cells, SR141716 failed to induce any kind of chromosome aberrations (CA). To assess whether SR141716 could induce a clastogenic effect

per se in the absence of the CB1R expression, CA also were evaluated 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 cytogenetic damage nor exhibiting genotoxic activity in normal cells.

Effect of SR141716 on Cell Cycle Progression. Because the inhibitory effect of SR141716 on MDA-MB-231 cell

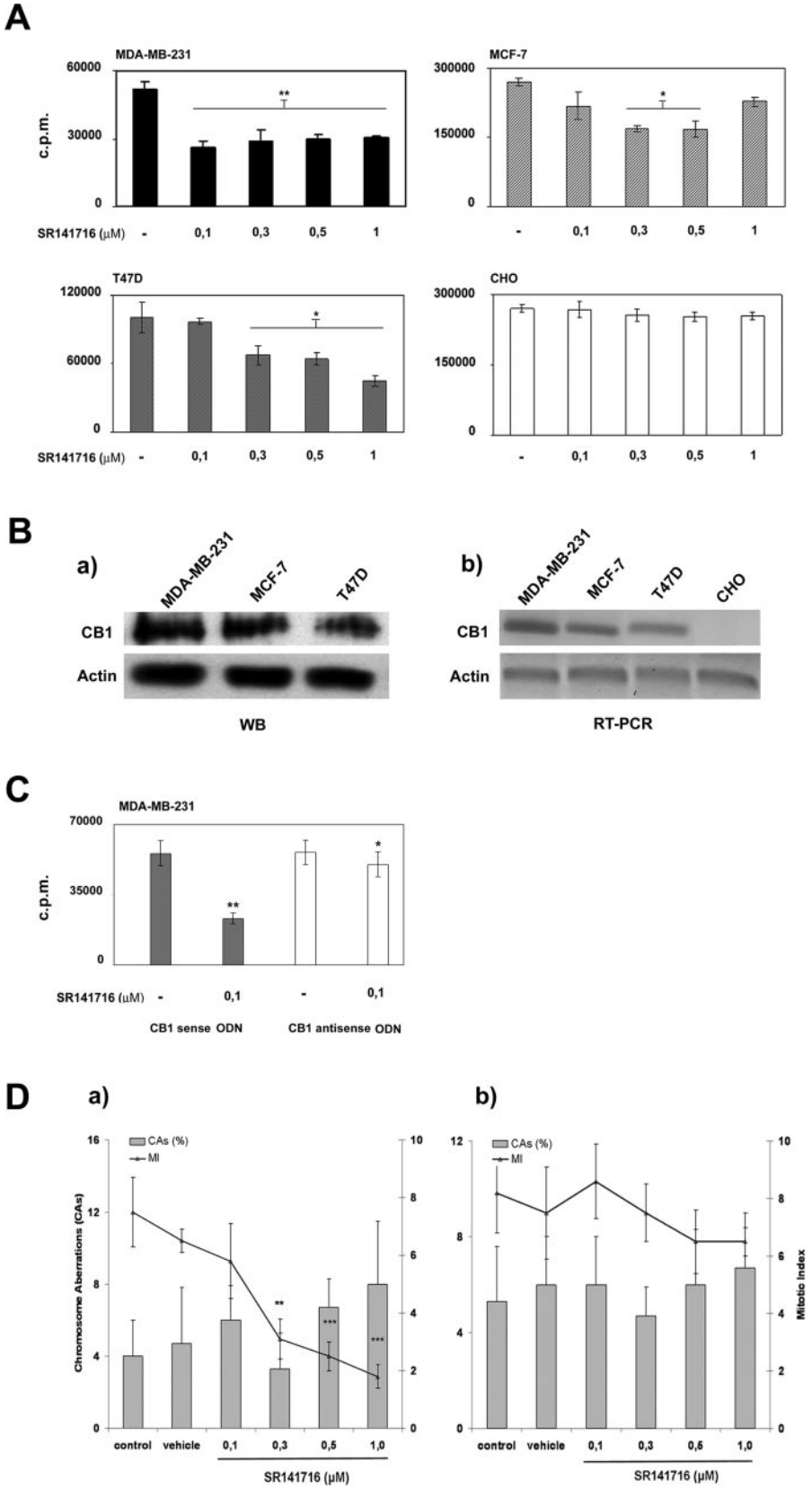

Fig. 1. Cell proliferation and cytogenetic effects of SR141716. A, MDA-MB-231, T47D, MCF-7, and CHO cells (5×10^4/well) were cultured in triplicate for 24 h with concentrations of SR141716 ranging from 0.1 to 1.0 μM. After 24-h incubation, [^{3}H]thymidine incorporation (0.5 μCi/well) was measured. The graphs report the mean of counts per minute ± S.E. 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 versus control; *, $p < 0.05$, **, $p < 0.01$). B, comparison of CB1R expression levels in the three breast cancer cell lines. a, cell lysates (50 μg of total proteins) were subjected to SDS-polyacrylamide gel electrophoresis. CB1R was revealed by Western blotting on nitrocellulose and hybridization with polyclonal antiactin antibody as loading control. The experiment was repeated three times. b, for RT-PCR the cells (MDA-MB-231, MCF-7, T47D, and CHO) were subjected to total RNA extraction, and RT was performed using Moloney murine leukemia virus reverse transcriptase, and random oligonucleotide primers for CB1R and actin were used. PCR were performed 30 s at 93°C, 1 min at 59°C, and 1 min at 69°C for 25 to 28 cycles to assess saturation of the signal. C, cell proliferation in MDA-MB-231 cells transfected with CB1R sense and antisense oligonucleotides as described under *Materials and Methods*. MDA-MB-231 cells (5×10^4/well) were cultured in triplicate for 24 h with 0.1 μM SR141716. After 24-h incubation, [^{3}H]thymidine incorporation (0.5 μCi/well) was measured. The graphs report the mean of counts per minute ± S.E. values of three independent experiments. Results were statistically significant [ANOVA, $p < 0.01$, with Student's t test SR141716 (sense ODN) versus control, **, $p < 0.01$, and SR141716 (antisense ODN) versus SR141716 (sense ODN), *, $p < 0.05$]. D, SR141716 is not genotoxic in normal cells. CA and mitotic index (MI) (determined as indicated under *Materials and Methods*) were evaluated in human lymphocytes (a) and in CHO cells (b) treated for 48 h with increasing concentrations of SR141716. The graphs report the mean ± S.E. values of three independent experiments. Results were statistically significant (ANOVA, $p < 0.01$, with Student's t test versus control, *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$).

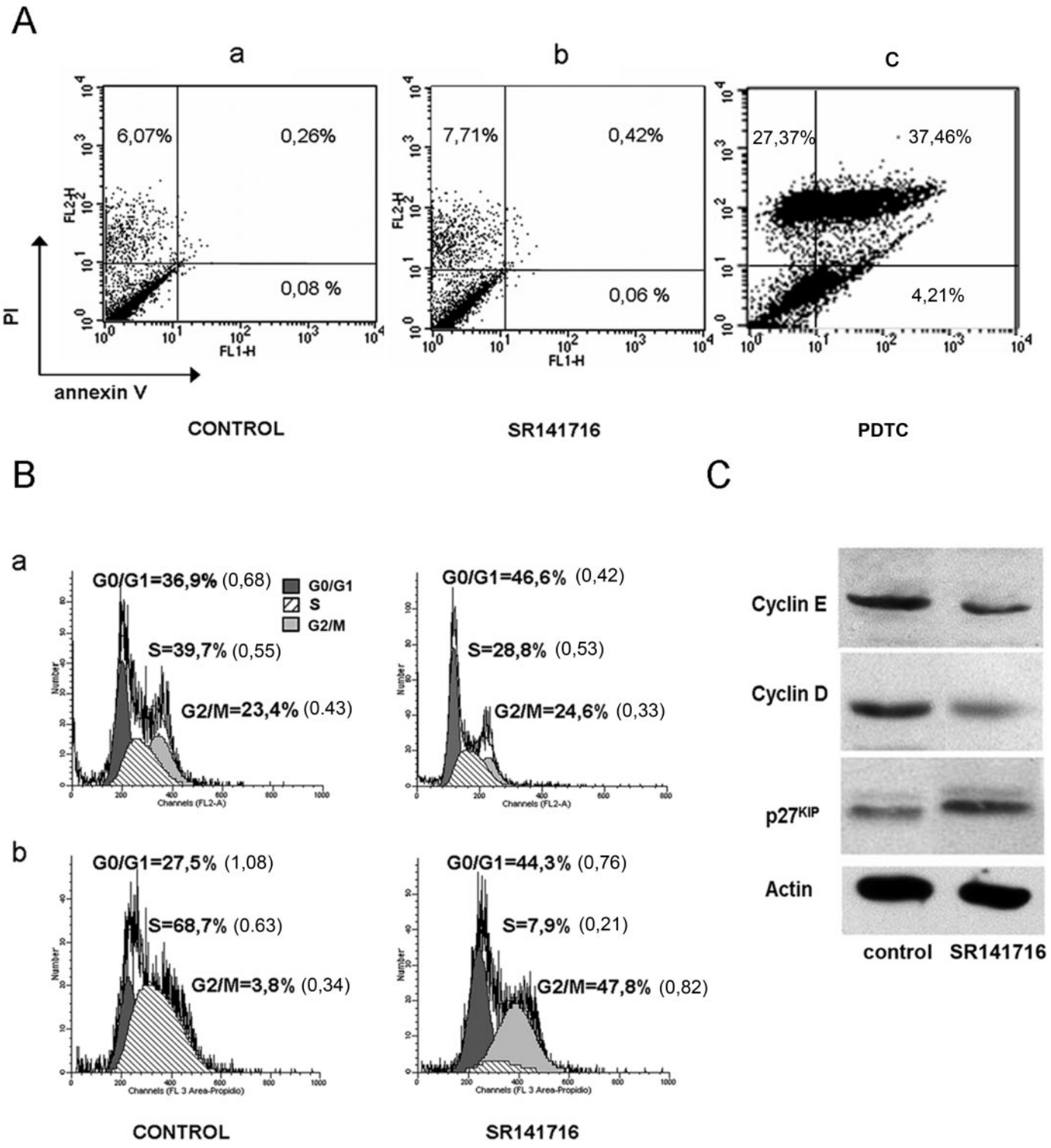

Fig. 2. Effect of SR141716 on apoptosis and cell cycle progression of MDA-MB-231 cells. A, MDA-MB-231 cells were incubated for 24 h with SR141716 0.1 μM; then cells were processed, and apoptotic cell death was analyzed as described under *Materials and Methods*. The panels relative to control (a) and SR141716 treatment (b) are representative of three independent experiments. As positive control of the procedure, in c the capacity of pyrrolidine dithiocarbamate 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 cells treated with SR141716 at 0.1 μM for 24 h were stained with propidium iodide and analyzed by flow cytometry. Data were subjected to ModFit analysis. Control and SR141716 panels show the relative percentage of cells in the G_0/G_1, S, and G_2/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 under *Materials and Methods* (in the section on Western blot), and an equal amount of total proteins (50 μg) was loaded on gel, resolved by SDS-polyacrylamide gel electrophoresis, and Western blotted with antibodies against the specific proteins. The same filters were stripped and reprobed with rabbit polyclonal antiactin antibody as loading control. Immunoreactive bands from three independent experiments were quantified using Quantity One program and normalized to actin bands.

proliferation was higher than that observed in less invasive and nonmetastatic cell lines, we further investigated the effects of SR141716 on this more invasive cell line, using 0.1 μM dose. The antiproliferative effect of SR141716 was not accompanied by apoptosis or necrosis as revealed by a flow cytometric assay with annexin V/propidium iodide double staining (Fig. 2A). To determine how SR141716 influences cell growth, cell cycle phase distribution of asynchronous cells was 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 G_0/G_1, S, and G_2/M phases of the cell cycle, respectively (Fig. 2Ba). We found that SR141716 induced a reduction in the percentage of cells in S phase (28.8%) with a parallel increase of cells in G_0/G_1 (46.6%) (Fig. 2Ba). This effect was also observed when cells were presynchronized by serum starvation. In this case, we found that SR141716 induced a strong increase in the percentage of cells in G_0/G_1 (44.3%) compared with the control (27.5%, Fig. 2Bb).

These results confirm the inhibition of proliferation at the G_1 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 G_1/S transition, Western blotting of cell lysates was carried out using immunodetection of various cell cycle-related proteins. Among these, cyclin D1 and E were reduced by SR141716 after 24-h treatment (Fig. 2C). In addition, as expected, the expression of cyclin-dependent kinase inhibitor $p27^{KIP1}$, which is known to mediate cell cycle arrest in response to various antiproliferative 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 2×10^6 MDA-MB-231 cells. When tumors were clearly detectable (15 days after injection), the mice were exposed to either vehicle or SR141716 (0.7 mg/kg/dose). Tumor growth was monitored, and the data revealed that exposure to SR141716 led to a significant decrease in tumor mass compared with vehicle control-treated mice (Fig. 3). Animals exhibited no outward signs of toxic or hypolocomotor effects, nor have effects of SR141716 (0.7 mg/kg/dose) on animal weight gain, hematocrit, or white blood cell levels been revealed (data not shown).

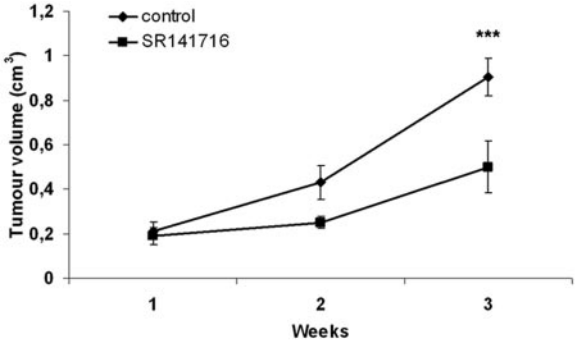

Fig. 3. SR141716 inhibits growth of xenograft tumors induced by MDA-MB-231 injection in mice. Tumor volume at different weeks from mice inoculation is shown. Data were shown as mean ± S.E. of $n = 5$ animals. Differences in tumor volumes after 3 weeks were significant ($p < 0.05$ by ANOVA followed by Bonferroni's test; ***, $p < 0.001$ for SR141716-treated group versus control group at 3 weeks).

Mechanism Underlying SR141716 Antiproliferative 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 to increase the efficiency and specificity of signal transduction by facilitating interactions between proteins and 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; Di Marzo et al., 2004; Grimaldi et al., 2006) and lipid rafts play a pivotal role in breast tumor cell invasion (Bourguignon et al., 2004), we decided first to check whether the CB1R associated with lipid rafts in the presence of its antagonist SR141716 and subsequently to investigate the role of raft microdomains in SR141716 signaling by using a cholesterol sequestering agent, MCD, which is known to perturb lipid raft composition (Sarnataro et al., 2002; Paladino et al., 2004).

Having previously documented and here reconfirmed (Fig. 4A) the enrichment of CB1R in lipid rafts/caveolae, we next examined the subcellular distribution of a signaling molecule relevant for CB1R signal transduction, ERK 1/2 and its phosphorylated form (pERK). As indicated in Fig. 4A, in control steady-state conditions (24 h) approximately 20% 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% CB1R and 3 to 5% total ERK 1/2 were present in lipid rafts, whereas, as predicted, pERK 1/2 was totally absent from them. These data suggest that after serum starvation and subsequent 15-min serum incubation, the composition of lipid rafts is perturbed, not completely restored, and consequently the association of the protein within is slightly affected compared with the control conditions (Fig. 4A, compare 24 h and 15 min, control).

To directly test the influence of SR141716 on the association of CB1R with lipid rafts, we included SR141716 (0.1 μM, 24 h) in the culture medium of MDA-MB-231 cells, and then cell lysates were subjected to OptiPREP density gradient flotation assay in TX-100 detergent. Pooled fractions 4 and 5 (representing the raft fractions, R) and 9 through 12 (representing nonraft fractions, NR) were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted to determine the relative abundance of proteins within. Incubation with SR141716 for 24 h (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 nonraft fractions, NR). The presence of a typical raft marker caveolin-1 (cav-1) (Bourguignon et al., 2004; Sarnataro et al., 2005) and of an extensively characterized raft marker GM1 (Bender et al., 2003; Paladino et al., 2004; Sarnataro et al., 2005) in the 5 to 30% OptiPREP gradient interface (lipid rafts, R) indicates that we successfully isolated rafts/caveolae microdomains. We also verified that a typical nonraft marker, the endoplasmic reticulum resident protein Bip/GRP78, was excluded from these fractions. It is noteworthy that the described effect of SR141716 was specific for the CB1R because cav-1 and the other molecules regularly distributed in the lipid raft fraction both in control and SR141716-treated samples (see Fig. 4, A and B, at 24 h). In light of these data, to understand whether the described effects of SR141716 may

have some implications at molecular signaling levels, we verified the expression levels of ERK 1/2 MAPK phosphorylated isoforms by checking with immunoblot analysis for pERK 1/2 and for the presence of CB1R in raft fraction after 15-min incubation with SR141716. As shown in the Fig. 4B, approximately 50% CB1R-enriched lipid rafts and, as well as in control conditions and previously shown in another cell type (Yang et al., 2004), 20% total ERK 1/2 were present in detergent-resistant domains (or rafts). Intriguingly, treatment with SR141716 for 15 min decreased the expression of pERK 1/2 compared with the control conditions.

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

To gain further insights into the involvement of lipid rafts in the antiproliferative effect induced by SR141716, MDA-MB-231 cell growth was tested after treatment with SR141716 (0.1 μM) in basal conditions and after perturbation of lipid raft 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 antiproliferative effect exhibited by SR141716 was almost completely reverted by pretreatment 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 receptor functions depend on the lipid raft integrity (Simons and Toomre, 2000; Simons and Ehehalt, 2002; Pike, 2003). It is noteworthy that as described previously in another cell line (Choi et al., 2004), MCD alone induced per se little antiproliferative effect.

Thus, to our surprise, CB1R antagonist SR141716, although it redistributes the receptor in the "nonraft" fractions, displays a significant antiproliferative 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

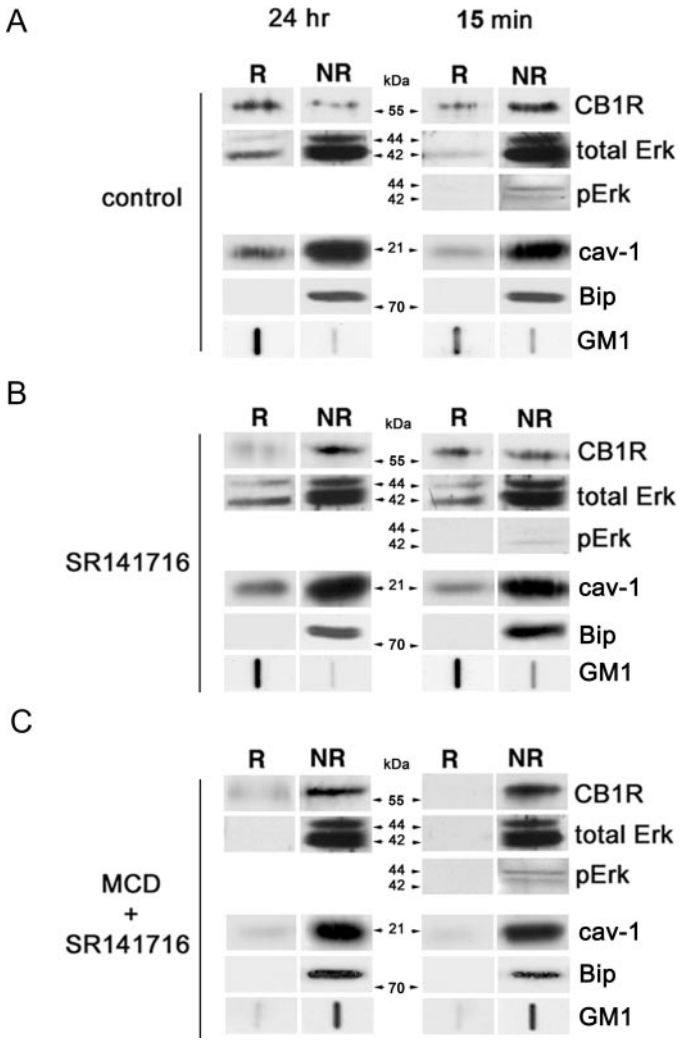

Fig. 4. Effect of SR141716 on CB1R raft association after lipid raft perturbation. MDA-MB-231 cells were incubated with indicated drugs for 24 h or for 15 min after serum starvation and lysed for 20 min in cold TNE/TX-100 buffer. Five hundred micrograms of total proteins were run through a "Two Step" OptiPREP 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 nonraft material (pooled fractions 9–12, NR) were loaded on 12% gel and revealed by Western blotting. Both at 24-h and 15-min serum incubation after starvation, the distribution of CB1R, the raft marker cav-1, and the nonraft protein Bip was analyzed by Western blot with the specific antibody and enhanced chemiluminescence. The distribution of GM1 was assessed on an aliquot of each fraction pool with dot blot analysis using Cholera Toxin-horseradish peroxidase. The levels of total and phosphorylated ERK 1/2 in R and NR fractions were analyzed by stripping nitrocellulose membrane and immunoblotting with the specific antibodies. Data were quantified by densitometric analysis with QuantityOne program. The relative percentages of CB1R, cav-1, and ERK 1/2 protein content in R and NR were expressed as percentage of total protein amount in each gradient fraction representing the 100%.

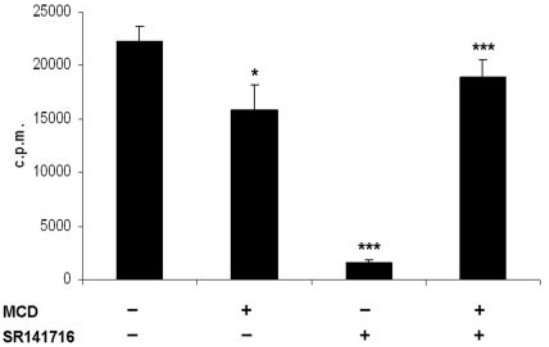

Fig. 5. Evaluation of MAPK activity after lipid raft perturbation. MDA-MB-231 cells were cultured in triplicate and treated with MCD for 15 min (alone or subsequently with SR141716 for 24 h) or for 24 h with SR141716, 0.1 μM. After 24-h incubation, [^{3}H]thymidine incorporation (0.5 μCi/well) was measured.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

integrity" to exert its antiproliferative property through CB1R signaling.

Discussion

The present findings provide unequivocal evidence for the role of the CB1R 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 CB1R, needs lipid raft/caveolae integrity to occur.

The endocannabinoid system is an almost ubiquitous signaling 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 signaling pathways (Bifulco and Di Marzo, 2002; Guzman, 2003). We have also previously reported that the endocannabinoid AEA 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 cell growth (Bifulco et al., 2001; Portella et al., 2003).

Because SR141716 functions as a specific CB1R antagonist, it could be expected that it would induce, if used alone, null or opposite effects to the antiproliferative activity exerted by CB1 agonist molecules (e.g., AEA). The absence of proliferation stimulation by SR141716 is, indeed, not surprising because it indicates that although endocannabinoids may help down-regulating tumor growth in vivo, this represents only one of many possible tumor-suppressing mechanisms occurring during cell transformation.

Indeed, we found that SR141716 has a significant and dose-dependent antiproliferative property, through the CB1R, in breast cancer cells such as T47D, MCF-7, and MDA-MB-231 cells. However, the inhibition of cell proliferation also seems to depend on the expression levels of the receptor in the different breast cancer cells used and on the less or more invasive phenotype of the breast cancer cell lines. Although SR141716 could exert the observed antiproliferative effect in MDA-MB-231 cells via a non-CB1R-dependent mechanism, results with antisense oligonucleotides provide more definitive evidence for the involvement of CB1R.

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. It is noteworthy that this concentration did not exert any cytotoxic effect in human lymphocytes. Because 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 antiproliferative 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 and Moore, 2005).

To gain further insight into the antiproliferative effect, we analyzed the cell cycle progression in SR141716-treated MDA-MB-231 cells. We observed that SR141716 is able to exert an antiproliferative effect by blocking the G_0/G_1 transition, similarly to what we had previously observed with endocannabinoids (e.g., AEA) 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 cyclin-dependent kinase 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 3 weeks of treatment; the overall observations suggest an antitumor activity of SR141716.

Furthermore, we studied in detail the molecular mechanism underlying SR141716 antiproliferative effect. MCD, a cholesterol sequestering agent that is known to perturb lipid raft composition, has also been described to have a cell growth inhibition effect in macrophage cells (Choi et al., 2004). Interestingly, MCD alone exhibits a small antiproliferative 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 the antiproliferative 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 (Bifulco and Di Marzo, 2002; Guzman, 2003), 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 a favorable platform to regulate CB1R signaling (McFarland et al., 2004; Bari et al., 2005). Given this information, we can speculate that the clear, albeit small, antiproliferative effect of MCD is probably 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 signaling deriving from SR141716 through CB1R interaction. As previously shown by Bouaboula et al. (1997), SR141716 inhibits MAPK activity only in cells overexpressing CB1R. Interestingly, we found that SR141716 decreases ERK 1/2 activity, and cholesterol depletion by MCD pretreatment reverts SR141716 inhibitory effects on ERK 1/2 in MDA-MB-231 cells (Fig. 4), indicating again that SR141716 signaling requires lipid raft integrity to occur and that the role of lipid rafts in the receptor-dependent signaling would be to render favorable the CB1R-ligand (SR141716) encounter on the cell surface, and/or at least the formation of the molecular machinery responsible for CB1-dependent signaling.

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

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