British Journal of Pharmacology (2008) 153, 1003–1010

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Rimonabant (SR141716) exerts anti-proliferative and immunomodulatory effects in human peripheral blood mononuclear cells

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Background and purpose: Rimonabant (SR141716) is the first selective cannabinoid receptor CB_1 antagonist described. Along with its anti-obesity action, emerging findings show potential anti-proliferative and anti-inflammatory action of SR141716 in several *in vitro* and *in vivo* models. In this study we have investigated the anti-proliferative and immunomodulatory effects of SR141716 in human peripheral blood mononuclear cells (PBMCs).
Experimental approach: We have evaluated *in vitro* the effect of SR141716 in human PBMCs stimulated with different mitogens. Cell proliferation was assessed by ^{3}H-thymidine incorporation. Cell cycle, cell death and apoptosis were analysed by flow cytometry. Protein expression was investigated by Western blot.
Key results: SR141716 significantly inhibited the proliferative response of PBMCs and this effect was accompanied by block of G_1/S phase of the cell cycle without induction of apoptosis and cell death. SR141716 used in combination with 2-methyl-arachidonyl-2′-fluoro-ethylamide (Met-F-AEA), a stable analogue of the endogenous cannabinoid anandamide, showed synergism rather than antagonism of the inhibition of cell proliferation. The immunomodulatory effects of SR141716 were associated with increased expression of IκB, phosphorylated AKT (p-AKT) and decreased expression of NF-κB, p-IκB, p-ERK, COX-2 and iNOS.
Conclusions and implications: Our findings suggest SR141716 is a novel immunomodulatory drug with anti-inflammatory properties.
British Journal of Pharmacology (2008) **153,** 1003–1010; doi:10.1038/sj.bjp.0707651; published online 24 December 2007

Keywords: rimonabant; immunomodulation; antiproliferative response; inflammation

Abbreviations: CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; COX-2, cyclooxygenase-2; ERK1/2, extracellular signal-regulated kinase 1/2; IκB, inhibitor of NF-κB; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; Met-F-AEA, 2-methylarachidonyl-2′-fluoro-ethylamide; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cell; PI, propidium iodide; SR141716, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl pyrazole-3-carboxamide; TNFα, tumour necrosis factor α

Introduction

Rimonabant (SR141716) (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4methyl pyrazole-3-carboxamide) is a potent and selective cannabinoid CB_1 receptor antagonist possessing food intake inhibiting and antiobesity activity (Rinaldi-Carmona *et al*., 1994, 1995; Colombo *et al*., 1998; Simiand *et al*., 1998; Ravinet Trillou *et al*., 2003; Carai *et al*., 2005; Jbilo *et al*., 2005). It is widely used as a tool to investigate the mechanisms by which cannabinoid agonists produce their pharmacological effects, and it may exert several intrinsic actions possibly by blocking the activation of cannabinoid CB_1 receptors by the endocannabinoid system, which is tonically activated under certain pathophysiological conditions (Di Marzo and Matias, 2005; Engeli *et al*., 2005; Matias *et al*., 2006). It has been previously described that SR141716 counteracts most of the antitumour effects of anandamide (AEA), suggesting that CB_1 receptors are uniquely involved in the effects of this compound (Bifulco *et al*., 2004; Grimaldi *et al*., 2006). Interestingly besides its antagonist properties, SR141716 also possesses inverse-agonist characteristics; it can block CB_1 receptor high constitutive activity at both levels of MAPK and adenylyl cyclase, in transfected CHO cells (Rinaldi-Carmona *et al*., 1994; Bouaboula *et al*., 1997; Hurst *et al*., 2002). It has been

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Received 21 September 2007; accepted 19 November 2007; published online 24 December 2007

reported that SR141716 can inhibit the differentiation of preadipocytes *in vitro* (Gary-Bobo *et al*., 2006). Furthermore, we observed a significant antitumour activity in tumour xenografts induced by the subcutaneous injection of KiMol cells (Bifulco *et al*., 2004). We have also demonstrated that SR141716 inhibits human breast cancer cells growth via a CB_1 receptor lipid rafts/caveolae-mediated mechanism (Sarnataro *et al*., 2006).

As CB_1 receptor expression has been detected outside the brain in many other tissues including immune system cells (Kaminski *et al*., 1992; Bouaboula *et al*., 1993; Schatz *et al*., 1997; Klein *et al*., 2003), recent papers on the role of endocannabinoids in the modulation of immune system have led to consideration of the therapeutic potential of SR141716 in inflammation. It was observed that SR141716 reduced nociceptive responses and inflammatory process in several experimental animal models. In rats and mice, it prevented the rise in TNF-α serum levels induced by *Escherichia coli* lipopolysaccharide and relieved neuropathic pain after sciatic nerve ligature (Smith *et al*., 2000; Costa *et al*., 2005). A role of SR141716 has also been proposed in immune-inflammatory pathogenic mechanisms (Croci *et al*., 2003). Oral administration of rimonabant dose-dependently prevented the indomethacin-induced small intestinal ulcers in rats. This effect was associated with a higher inhibition of TNF-α levels and myeloperoxidase activity compared with the selective CB_2 receptor antagonist SR144528. SR141716 produced similar inhibitory effects also in CB_1 receptor knockout mice, suggesting an antiulcerogenic action not relying on CB_1 antagonism. However, in the same CB_1 knockout mice, SR141716 failed to counteract the increased lipopolysaccharide-induced TNF-α plasma levels that were CB_1 receptor dependent. This finding suggests different mechanisms of action of SR141716 in the modulation of the inflammatory process. Further, recent studies suggested the potential of SR141716 for the treatment and prevention of chronic inflammatory diseases. It was demonstrated that SR141716 reduced joint inflammation in a model of adjuvant-induced unilateral arthritis in obese rats and inhibited thermal and mechanical hyperalgesia in obese as well as in lean arthritic rats (Croci and Zarini, 2007).

In this study, we have assessed the antiproliferative and immunomodulatory properties of SR141716 in human peripheral blood mononuclear cells (PBMCs). The inhibition of cell proliferation was not accompanied by cell death or induction of apoptosis but was associated with a block of G_1/S phase transition of the cell cycle. An additive/synergistic effect of SR141716 and 2-methyl-arachidonyl-2′-fluoro-ethylamide (Met-F-AEA) was observed on cell proliferation. The mechanism through which SR141716 exhibited its effects relied on the modulation of T-cell activation pathways, upregulation of inhibitor of NF-κB factor kinase (IκB) and phosphorylated AKT (p-AKT); downregulation of nuclear factor κB (NF-κB), phosphorylated IκB (p-IκB), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), reduced expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were also observed. Our data demonstrate that SR141716 *in vitro* has antiproliferative and immunomodulatory effects, suggesting potential anti-inflammatory properties.

Methods

Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells derived from buffy coats of peripheral blood taken from healthy volunteers were isolated by density gradient centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway). The cells were washed three times with phosphate-buffered saline (PBS) and resuspended in culture medium RPMI, 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St Louis, MO, USA). All assays were performed in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with penicillin/streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies) and 10% heat-inactivated fetal calf serum.

Proliferation assays on human PBMCs

Isolated PBMCs (10^5 cells per well) were cultured in triplicate in round-bottomed 96-well plates in a final volume of 200 μl of RPMI 10% fetal calf serum. Cells were stimulated with supernatants containing anti-CD3 from OKT3 hybridoma (1:200 final dilution) or phytohaemagglutinin ($4\,\mu g\,ml^{-1}$) (Sigma). SR141716 dissolved in DMSO was added to the cells to achieve final concentrations of 0.3, 1, 3 and 10 μM. Under the same experimental conditions, cells were stimulated with OKT3 supernatants in the presence of 10 μM Met-F-AEA, dissolved in ethanol and SR141716 at final concentrations of 0.3 and 1 μM; the vehicle was used as control in all the experiments. After 48 h of incubation at 37 °C, cells were pulsed with 1 μCi of ^{3}H-thymidine (Amersham-Pharmacia Biotech, Cologno Monzese, Milano, Italy) and harvested after a further 18 h of incubation. Radioactivity was measured in a scintillation counter (Wallac, Turku, Finland).

Flow cytometry assays

To quantify apoptosis, cell death and cell cycle progression, 10^6 PBMCs were cultured with OKT3 supernatant (1:200 dilution) and SR141716 at 10 μM in RPMI 10% fetal calf serum for 48 h at 37 °C in 24-well plates. To detect apoptosis, cells were double stained with Annexin V-FITC (Pharmingen, San Diego, CA, USA) and propidium iodide (PI) (Sigma). After 10 min of incubation at 4 °C, cells were washed twice with PBS, resuspended in Annexin V buffer and stained with Annexin V-FITC. PI was added to the cells before flow cytometric analysis. 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 resuspended in 300 μl of PBS; 700 μl of 70% ethanol were added slowly to the cells on a vortex and kept at −20 °C for 1 h. PI ($10\,\mu g\,ml^{-1}$) in PBS containing $100\,U\,ml^{-1}$ DNase-free RNase was added to the cells (Nicoletti *et al*., 1991); 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, USA) program. Each sample was analysed using 10 000 events corrected for debris and aggregate populations.

Evaluation of the combined effects of SR141716 and Met-F-AEA
The following equation described by Chou and Talalay (1984) (Chou *et al*., 1994) was used to evaluate the nature of the interaction between SR141716 and Met-F-AEA:

$$f_a/f_u = (D/D_m)^m$$

where D = the dose of drug; D_m = the dose required to produce the median effect (analogous to the more familiar IC_{50} values); f_a = the fraction affected by the dose; f_u = the fraction unaffected ($f_u = 1 - f_a$); and m is a Hill-type coefficient signifying the sigmoidicity of the dose–effect curve.

We obtained the confidence interval (CI) values using the Biosoft CalcuSyn written in BASIC for automatic graphing of CI with respect to FA. The two drugs were either mutually non-exclusive or mutually exclusive. When $CI < 1$, synergism is indicated. When $CI = 1$, summation is indicated. When $CI > 1$, antagonism is indicated.

Electrophoresis and immunoblots
Cell extracts were prepared from human PBMCs. Cells were activated with OKT3 supernatant (1:200 dilution) and treated with SR141716 at 10 and 3 μM. After 48 h of incubation, cells were washed twice with PBS, resuspended 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. Following cell incubation, the preparation of samples for NF-κB analysis was processed to separate cytoplasmic and nuclear fractions according to the instructions reported in the Chemicon's Nuclear Extraction Kit (catalog no. 2900). Supernatants were collected and the protein concentration evaluated by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein extracts (40 μg) were boiled in Laemmli 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 CB_1 (Santa Cruz Biotechnology Inc.) IκB, NF-κB (Santa Cruz Biotechnology Inc.), phospho-IκB (Ser 32; Santa Cruz Biotechnology Inc.), phospho-AKT (Ser 473; Cell Signalling), phospho-ERK1/2 (Cell Signalling), iNOS (Santa Cruz Biotechnology Inc.) and COX-2 (Chemicon). Immunodetection of specific proteins was carried out with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Bio-Rad), using the enhanced chemiluminescence (ECL) system (Amersham, GE Healthcare, Buckinghamshire, UK).

Statistical analyses
Analyses were performed using ANOVA followed by the Bonferroni *post hoc* analysis for multiple comparisons. Results are expressed as means ± s.d.; *P*-values less than 0.05 were considered to be statistically significant.

Drugs
SR141716 was kindly donated by Sanofi-Aventis Research (Montpellier, France). Met-F-AEA was purchased from Calbiochem, San Diego, CA, USA.

Results

SR141716 effect on PBMC proliferation
The effect of SR141716 was investigated in human PBMCs activated by OKT3 supernatants or by phytohaemagglutinin. Cells were cultured with SR141716 at the concentrations of 0.3, 1, 3 and 10 μM for 48 h. SR141716 inhibited the proliferation of OKT3-activated PBMCs in a concentration-dependent manner. The inhibitory effect was significant at the concentrations of 3 and 10 μM when cells were activated with OKT3, whereas significant inhibition was observed at 10 μM when cells were stimulated with phytohaemagglutinin (Figures 1a and b).

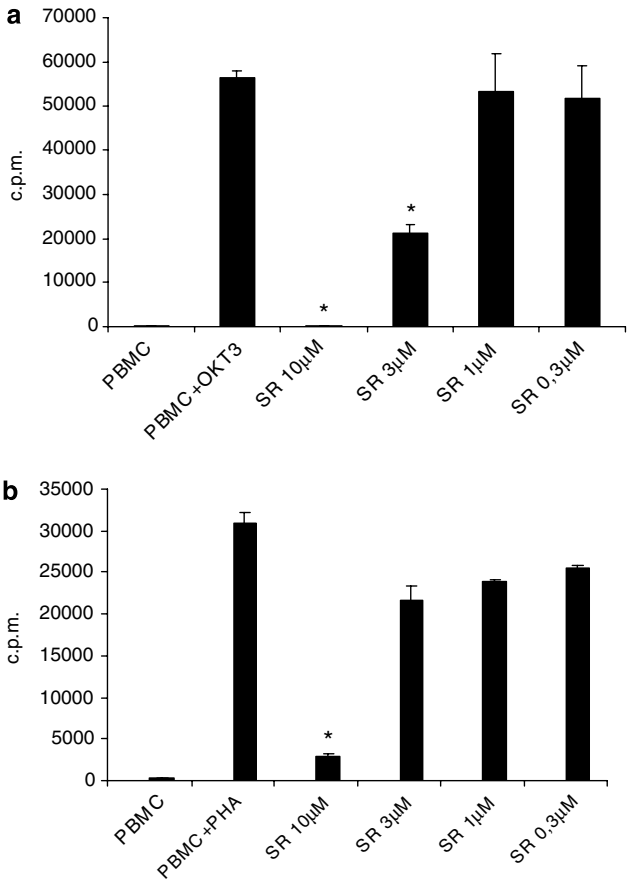

Figure 1 SR141716 inhibited proliferation of peripheral blood mononuclear cell (PBMCs). Activated PBMCs (10^5 cells per well) were treated with SR141716 at the indicated concentrations, in triplicate, for 48 h. Proliferation was measured after 18 h of ^{3}H-thymidine incorporation (1 μCi). PBMCs were activated with OKT3 supernatant (**a**) or phytohaemagglutinin (PHA) (**b**). The counts per minutes (c.p.m.) of representative experiments out of five are shown. The statistical significance is indicated in the graphs (*$P < 0.05$ calculated with respect to the activated cells).

SR141716 effect on cell cycle, death and apoptosis

To investigate whether the inhibitory effect of SR141716 was associated with changes in the cell cycle progression, cell death or induction of apoptosis, we performed flow cytometry analysis. PBMCs treated with SR141716 at 3 and 10 μM for 48 h were stained with PI and analysed by flow cytometry. The results obtained indicated that SR141716 blocked the G_1/S transition of the cell cycle progression (Figure 2). The effect is represented as decreased percentage of S phase with respect to the control OKT3-activated cells. Furthermore, to address whether the effect of SR141716 on cell proliferation could be related to toxicity and subsequent cell death, we stained PBMCs with Annexin V-FITC and PI to analyse the induction of apoptosis. OKT3-activated PBMCs were treated with SR141716 at 3 and 10 μM for 48 h. The flow cytometric analysis was performed on gated lymphocytes. We plotted the percentage of double-positive cells stained for Annexin V and PI, and no significant difference between treated and untreated OKT3-activated PBMCs was observed (Figure 3). We found that the percentage of Annexin V-positive cells and PI-positive cells treated with SR141716 was not significantly different from the control untreated OKT3-activated cells.

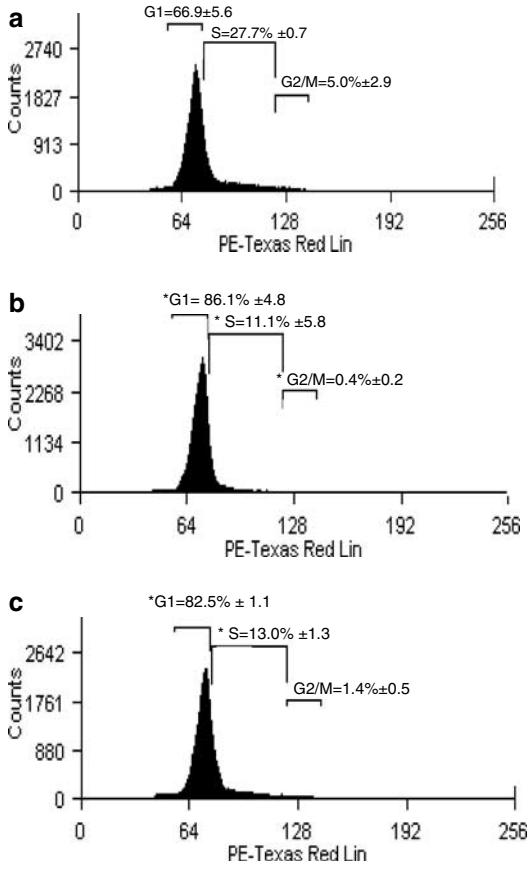

Figure 2 SR141716 blocked the G_1/S phase transition of the cell cycle. Histogram (**a**) represents the cell cycle progression of OKT3-activated peripheral blood mononuclear cells (PBMCs); histogram (**b**) represents the cell cycle progression of OKT3-activated PBMCs treated with SR141716 at the concentration of 10 μM. (**c**) Represents the cell cycle progression of OKT3-activated PBMCs treated with SR141716 at the concentration of 3 μM. The numbers of the cells counted are shown on the Y axes. The percentage of cells in each phase of the cell cycle of three independent experiments is shown (*$P<0.05$ with respect to untreated OKT3-activated cells). The histograms reported are representative of three independent experiments performed in triplicate.

Additive/synergistic effect of SR141716 combined with Met-F-AEA

To assess combinatory effects, OKT3-activated PBMCs were exposed for 48 h to SR141716 and Met-F-AEA, the metabolically stable endocannabinoid analogue of anandamide. Met-F-AEA was used at 10 μM, a dose that we previously reported to be inhibitory in this system (Malfitano *et al*., 2006). SR141716 was used alone or in combination with Met-F-AEA at the concentrations of 0.3, 1, 3 and 10 μM. The proliferation–inhibitory effect was assessed by the ^{3}H-thymidine incorporation assay (Figure 4a). Then, the inhibition rates were analysed by the method of Chou and Talalay (1984). Briefly, assessment of drug interaction was carried out as described in the Methods by calculating CI. CI/fractional effect curves represent the CI vs the fraction of cells affected/killed by SR141716 and Met-F-AEA in combination (Figure 4b).

The experiments were repeated at least in triplicate. As shown in Figure 4b, the CI values were 1 at the doses of SR141716 of 0.3 and 1 μM (1 and 2 in the graphs, respectively), whereas the CI values were <1 at the doses of SR141716 of 3 and 10 μM (3 and 4 in the graphs, respectively). The results reported indicated that SR141716 and Met-F-AEA have an additive effect at low doses and a synergistic effect at high doses on inhibiting proliferation of PBMCs.

SR141716 inhibited NF-κB, p-IκB, p-ERK1/2, COX-2, iNOS and increased IκB, and p-AKT expression

To address whether SR141716 could interfere with the T-cell activation pathway, we analysed in cell extracts, the

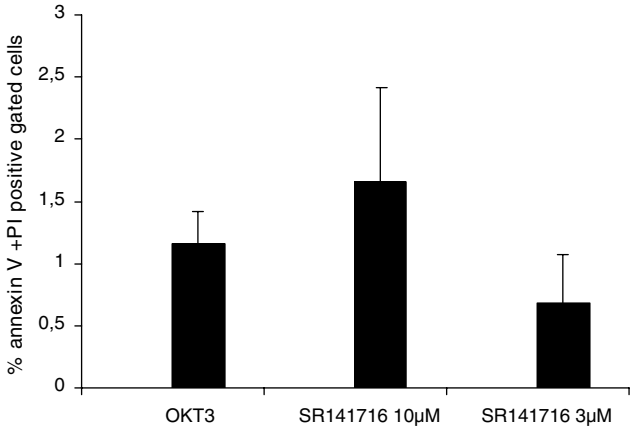

Figure 3 SR141716 did not induce apoptosis in peripheral blood mononuclear cells (PBMCs). Histogram shows the mean of four independent experiments of Annexin V and propidium iodide (PI) staining of OKT3-activated PBMCs in the presence or in the absence of SR141716 at the indicated concentrations.

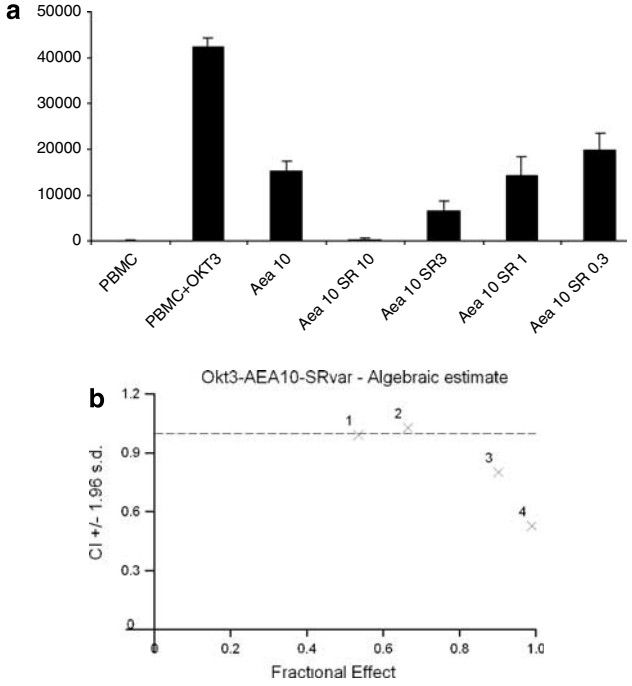

Figure 4 *In vitro* additive/synergistic antiproliferative effects of SR141716 combined with 2-methylarachidonyl-2′-fluoro-ethylamide (Met-F-AEA). OKT3-activated peripheral blood mononuclear cells (PBMCs; 10^5 cells per well) were cultured for 48 h and treated with Met-F-AEA at 10 μM in the presence or in the absence of SR141716 at the concentrations of 0.3 μM (1), 1 μM (2), 3 μM (3) and 10 μM (4). Proliferation was measured after 18 h of ^{3}H-thymidine incorporation (1 μCi). The related representative histogram is shown as means ± s.d. (**a**) The confidence interval (CI) values were determined as described in the Methods, and the combination analysis was done using the CalcuSyn software. CI/fractional effect curves showed the CI vs the fraction of cells affected/killed by SR141716 and Met-F-AEA in combination. (**b**) Combinations were additive when CI = 1, synergistic when CI < 1 and antagonist for CI > 1.

expression of NF-κB in the cytosolic and nuclear fractions, IκB, p-IκB, p-AKT and p-ERK1/2 after 48 h of incubation. We detected increased expression of IκB after SR141716 treatment at the concentrations of 3 and 10 μM with respect to untreated OKT3-activated cells. This modulation correlates with decreased expression of p-IκB and NF-κB following SR141716 treatment and increased level in untreated OKT3-activated cells (Figure 5). As AKT has been associated with resistance to apoptosis (Lawlor and Rotwein, 2000), we assessed whether the expression levels of the phosphorylated protein kinase, p-AKT, was altered following treatment with SR141716. We found increased expression levels of p-AKT (as assessed by western blotting) when cells were treated with SR141716 (Figure 5). These data correlate with the resistance to apoptosis that we have shown here. Furthermore, we detected that SR141716 is involved in the MAP kinase pathway as a significantly reduced expression of p-ERK1/2 was observed following SR141716 treatment in activated PBMCs. To investigate potential anti-inflammatory action of SR141716, we analysed the expression of COX-2 and iNOS, known targets of NF-κB. After 48 h of incubation, cell extracts were prepared from OKT3-activated PBMCs in the presence or in the absence of SR141716 at the concentrations of 3 and 10 μM. We found a decreased expression of COX-2 and iNOS after SR141716 treatment with respect to untreated OKT3-activated cells (Figure 5).

Discussion

This study represents the first evidence showing that SR141716 exerts immunomodulatory effects in normal human cells *in vitro*, modulating the response of human, mitogen-activated PBMCs through inhibition of cell proliferation. The immunosuppressive effect of SR141716 in OKT3-activated PBMCs was associated with the block of G_1/S phase transition of the cell cycle, which was not accompanied by induction of apoptosis and cell death, thus suggesting that SR141716 is not toxic in this system. We previously reported CB_1 receptor-dependent antiproliferative properties of SR141716 in tumour epithelial cells at low doses that were inhibitory in cancer cells and not cytotoxic in human lymphocytes (Sarnataro *et al*., 2006). The mechanism of SR141716 immunomodulation in the activated cells does not seem to involve modulation of the CB_1 receptor, as we found no regulation of the expression of CB_1 receptors after SR141716 treatment at the concentrations of 10 and 3 μM, with respect to the similar expression observed in the activated and non-activated cells (data not shown). To assess combinatory effects of synergism, summation or antagonism of SR141716 with CB_1 receptor agonist immunosuppressive drugs, we analysed the combined effect of SR141716 with Met-F-AEA. We selected Met-F-AEA as it is a more stable anandamide analogue with respect to anandamide, and also because it has been previously used to observe potential counteracting effects of SR141716 in epithelial cells (Bifulco *et al*., 2004; Grimaldi *et al*., 2006). We detected an additive effect at low doses of SR141716, and a synergistic effect at high doses of SR141716 in the inhibition of cell proliferation obtained by the combination of SR141716 and Met-F-AEA. This finding demonstrates that SR141716 in human mitogen-activated PBMCs shows additive/synergistic characteristics, not behaving, as previously observed in epithelial cells, as an antagonist for Met-F-AEA. It has been previously suggested that SR141176, besides its antagonist properties, also functions as an inverse agonist, because it can block CB_1 receptor high constitutive activity and transduction pathways of coupled tyrosine kinase receptors, such as growth factor receptors (Bouaboula *et al*., 1997).

To address the effect produced by SR141716 on transduction pathways activated through stimulation of CD3–T-cell receptors (TCRs) by OKT3 mitogens, we analysed as a downstream indicator, the transcription factor NF-κB, to further support the hypothesis of inverse agonist behaviour of SR141716 in cells of the immune system. In particular, we proposed that SR141716 acted via its involvement in the regulation of NF-κB activation, one of the key regulator of essential genes for lymphocyte activation and generation of immune and inflammatory response (Kane *et al*., 2000; Karin and Ben-Neriah, 2000). In the canonical pathway, which is activated in T cells by TNFα or by TCR/CD28 signalling, phosphorylation by IκB kinases and degradation of IκB trigger the translocation of NF-κB from the cytoplasm to the

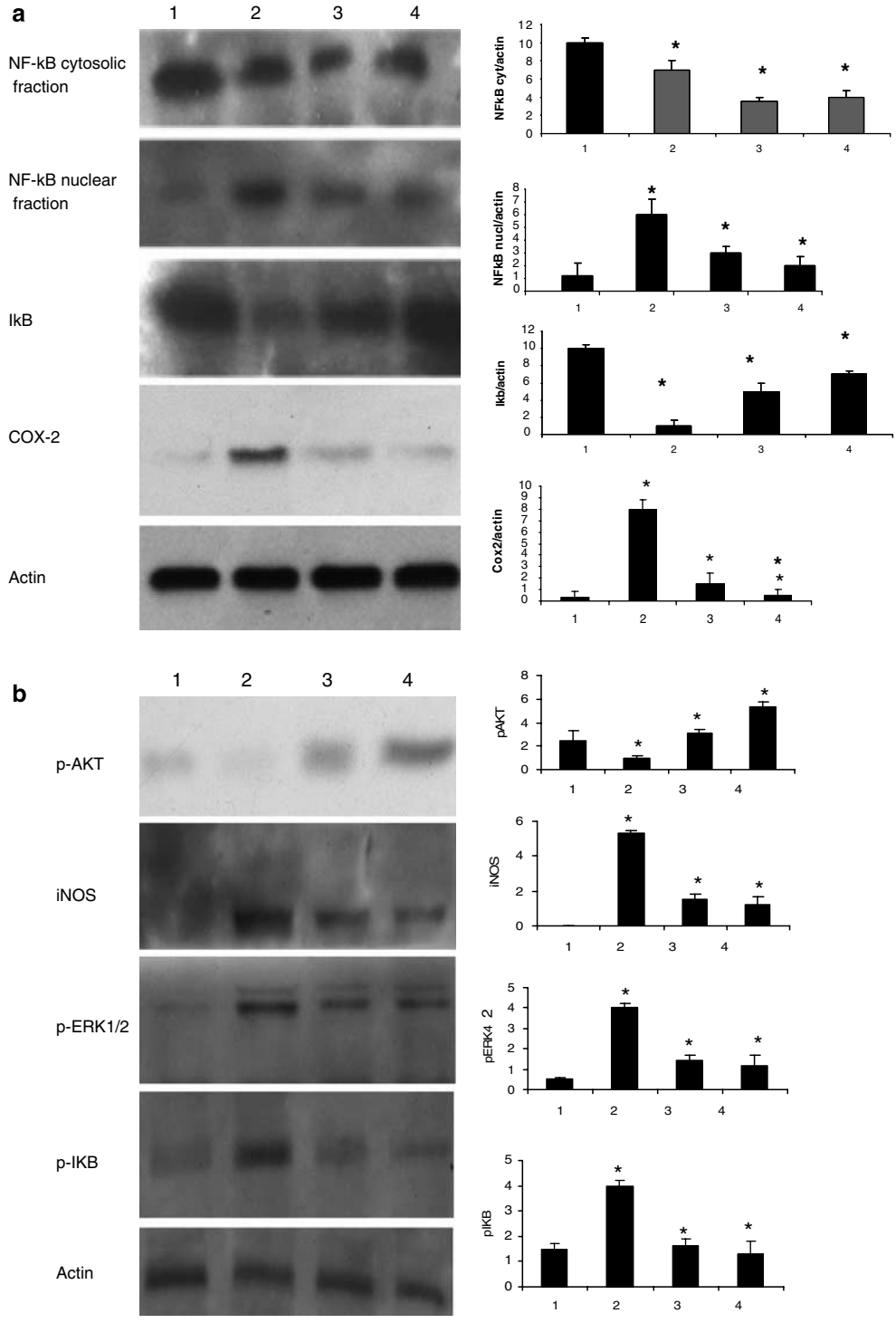

Figure 5 SR141716 inhibited nuclear factor κB (NF-κB), cyclooxygenase-2 (COX-2), inhibitor of NF-κB (IκB), inducible nitric oxide synthase (iNOS) and phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), and increased phosphorylated p-IκB (p-IκB) and phosphorylated AKT (p-AKT) expression. In (**a**), western blots of the expression of IκB, COX-2, and cytosolic and nuclear fractions of NF-κB in lysates of PBMCs and OKT3-activated PBMCs in the presence or in the absence of SR141716, normalized by actin. In (**b**), western blots of the expression of p-AKT, iNOS, p-ERK1/2 and p-IκB in lysates of PBMCs and OKT3-activated PBMCs in the presence or in the absence of SR141716, normalized by actin. In both (**a**) and (**b**), lane numbering is as follows: lane 1, PBMCs; lane 2, OKT3-activated PBMCs; lane 3, OKT3-activated PBMCs in the presence of 3 μM of SR141716; lane 4, OKT3-activated PBMCs in the presence of 10 μM of SR141716. The experiments were repeated at least three times. The histograms show the means ± s.d. of at least three independent experiments. Intensity of immunoreactive bands, calibrated to the intensity of tested proteins, were quantified using Quantity One program. In (**b**), the more intense band of the p-ERK doublet (42 kDa) was quantified. *$P<0.01$ calculated for OKT3-activated PBMCs vs PBMCs, for OKT3-activated PBMCs in the presence of 3 μM of SR141716 vs PBMCs and for OKT3 activated PBMCs in the presence of 10 μM of SR141716 vs PBMCs.

nucleus (Karin and Ben-Neriah, 2000). We showed that SR141716 interfered with the T-cell activation-signalling cascade by modulating IκB and NF-κB. We found that SR141716 upregulated IκB in OKT3-activated PBMCs with respect to untreated OKT3-activated cells and that this finding correlated with the observed downregulation of p-IκB and NF-κB, in the cytosolic and, more clearly, in the nuclear fractions. We also found that SR141716-induced phosphorylation of the protein kinase, PKB/AKT. This result correlates with our present data showing that cells treated with SR141716 do not undergo apoptosis, and is supported by previous findings demonstrating that the AKT-signalling pathway is responsible for cell survival (Lawlor and Rotwein, 2000).

We previously reported that Met-F-AEA and arvanil, a synthetic hybrid molecule between anandamide and capsaicin (Melck *et al*., 1999), exert immunoregulatory effects on human mitogen-activated PBMCs, inhibiting proliferation with the block of the G_1/S phase transition of the cell cycle and activation of p-AKT without induction of apoptosis (Malfitano *et al*., 2006, 2007). Furthermore, it was reported that anandamide and arvanil prevent TNFα-induced degradation of IκB, and NF-κB activation in the 5.1 cell line (Sancho *et al*., 2003; Márquez *et al*., 2006). The effect of Met-F-AEA was CB_1 receptor independent, as SR141716 pretreatment before Met-F-AEA exposure did not prevent NF-κB activation. We here report that SR141716 in human PBMCs exerts the same effects as Met-F-AEA and arvanil, even showing additivity/synergism with Met-F-AEA. These findings further suggests that in mitogen-activated cells of the immune system, SR141716 has agonist-like properties rather than antagonist effects, as confirmed by inhibition of NF-κB, a downstream target of TCR activation. Effective T-cell activation requires two signals: TCR engagement by a peptide/MHC complex and costimulation through several accessory molecules. A key element in the TCR-signalling pathway involved in transducing receptor-initiated signals to the nucleus is the family of mitogen-activated protein (MAP) kinases. The best-studied component of these kinases in TCR signalling is ERK1/2 (Whitehurst *et al*., 1992).

The possible mechanisms underlying the inhibitory effect of SR141716 on T-cell activation were further explored. We found that SR141716 appears to play a role in the T-cell activation pathway preventing the activation of the signal transducer p-ERK1/2, thus suggesting that SR141716 may influence events in the MAP kinase pathway involved in TCR-signalling transductions.

The transcription of pro-inflammatory cytokines, chemokines, cytokine receptors, adhesion molecules and key enzymes in the inflammatory process, such as COX-2 and iNOS, is regulated by NF-κB (Ghosh *et al*., 1998) that has been demonstrated to be highly activated at sites of inflammation in several diseases (Tak and Firestein, 2001). We assessed whether SR141716 could exhibit not only immunomodulatory effects but also potential anti-inflammatory action by evaluating the expression of COX-2 and iNOS. We detected decreased expression of COX-2 and iNOS following SR141716 treatment with respect to the untreated OKT3-activated cells, thus suggesting an inhibitory role of SR141716 in inflammatory conditions. This result is in agreement with the observed modulation of NF-κB by SR141716, as the block of NF-κB activity could prevent the transcription of the enzymes COX-2 and iNOS. The inhibition of expression of COX-2 and iNOS also correlates with previous studies showing anti-inflammatory properties of SR141716. It was reported that SR141716 reduced joint inflammation in a model of adjuvant-induced unilateral arthritis in obese rats (Croci and Zarini, 2007).

In conclusion, the present study shows antiproliferative and immunomodulatory effects of SR141716. SR141716 markedly reduced proliferation of human PBMCs, blocking the G_1/S phase transition of the cell cycle without induction of apoptosis or cell death and with activation of p-AKT and IκB. The mechanisms of immunomodulation by SR141716 involve downregulation of NF-κB, p-IκB and p-ERK. These findings and the additive/synergistic effect observed with the combination with Met-F-AEA, strongly suggest an agonist-like effect of SR141716 in mitogen-stimulated human PBMCs. Furthermore, the inhibition of COX-2 and iNOS expression, suggests a potential use of SR141716 to relieve inflammatory symptoms and therapeutic application in the treatment of inflammatory diseases.

Acknowledgements

This study was supported by Sanofi-Aventis (grant to MB) and by the Associazione Educazione e Ricerca Medica Salernitana, ERMES.

Conflict of interest

The authors state no conflict of interest.

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