SPANDIDOS PUBLICATIONS

Synergistic inhibition of human colon cancer cell growth by the cannabinoid CB1 receptor antagonist rimonabant and oxaliplatin

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Abstract. Rimonabant (SR141716), a highly selective cannabinoid receptor antagonist, exerts along with its anti-obesity action, pleiotropic functions affecting a broad range of diseases, from obesity-related co-morbidities to drug dependence and cancer. Several studies suggested an anti-tumour activity of rimonabant in several *in vitro* and *in vivo* models. In this study, we compared the anti-proliferative effect of SR141716 in the human colon cancer cell line DLD-1 with oxaliplatin, one of the cytotoxic drugs currently used in the treatment of colorectal cancer. We show that SR141716 inhibits DLD-1 cell proliferation similarly to oxaliplatin and if administered in combination SR141716 potentiated the inhibitory effect caused by oxaliplatin. Assessment of drug interaction was performed calculating combination index that showed a strong synergistic effect between the two drugs added to cells in combination. Our findings suggest that the combined synergic effect of SR141716 and oxaliplatin improves the blocking of colon cancer cell proliferation. Therefore, this combination merits further explorations in preclinical and clinical settings.

Introduction

Rimonabant (SR141716) (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4methyl pyrazole-3-carboxamide) is a potent and selective cannabinoid receptor (CB1) antagonist possessing food intake inhibiting and anti-obesity activity (1-7). It also shows a number of beneficial pharmacological effects in several pathological situations, i.e. metabolic syndrome, diabetes, nicotine dependence (8). So far, a number of novel biological effects of SR141716 *in vitro* and *in vivo* have been reported. Among others, it shows anti-fibrotic effect in response to acute liver injuries (9) and a systemic anti-inflammatory activity in indomethacin-induced small intestinal ulcers in rats (10). SR141716 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 CB1 receptors by the endocannabinoid system, which is tonically activated under certain pathophysiological conditions (11-13). It has been previously described that SR141716 but not the CB2 receptor antagonist counteracts most of the anti-tumour effects of anandamide (AEA), suggesting that CB1 receptors are uniquely involved in the effects of this compound (14,15). Interestingly, besides its antagonist properties, SR141716 possesses also inverse-agonist characteristics (1,16,17) exhibiting a significant anti-tumour effect in tumour xenografts induced by the subcutaneous injection of KiMol cells (15). We have also recently shown that 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 anti-proliferative effect of rimonabant 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 kinase inhibitor p27KIP1. SR141716 exerted a significant anti-proliferative action, *in vivo*, reducing the volume of xenograft tumours induced by MDA-MB-231 injection in mice. On the other hand, at the concentration range in which we observed the anti-proliferative effect in tumour cells, we did not observe evidence of any genotoxic effect on normal cells (18).

Numerous clinical studies combining the new generation of targeted therapies and chemotherapy have had mixed results. Preclinical studies are useful to identify potential

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Abbreviations: CI, combination index; AEA, anandamide; VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; DRI, dose reduction index

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antagonism/synergy between certain drugs, with the potential to predict the most efficacious combinations for further application in the clinical setting. Several studies have been performed combining new molecules with the currently available chemotherapeutic drugs.

Oxaliplatin is a chemotherapy agent that is given as treatment for certain types of cancer. It is commonly indicated in the treatment of colorectal cancer in both adjuvant and advanced settings. The treatment of colorectal cancer has consisted of fluoropyrimidine-based chemotherapy for over 50 years. The addition of irinotecan and oxaliplatin to fluoropyrimidines has become the standard of care for the treatment of metastatic colorectal cancer. These regimens have significantly increased both the time to progression and the overall survival in patients with metastatic colorectal cancer. It has been very reported that oxaliplatin has synergistic effects in combination with ZD6474 (Zactima), an inhibitor of vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) and RET tyrosine kinase activity. In fact, ZD6474 in combination with oxaliplatin is synergistic when oxaliplatin is administered before ZD6474 in colorectal cancer cells *in vitro* (19). Furthermore, combined exposure to ZD1839 (Iressa), a novel EGFR tyrosine kinase inhibitor and oxaliplatin also exerted synergy in colorectal cancer cell lines (20).

In this study, we compared the anti-proliferative effect of SR141716 on the human colon cancer cell line DLD-1 with oxaliplatin. We carried out combinatory studies of cell proliferation to assess the efficacy of both drugs simultaneously used in DLD-1 cells. By the combination of both drugs we detected synergism associated with higher anti-tumour effect of oxaliplatin at lower inefficacious doses.

Materials and methods

Drugs. Oxaliplatin and SR141716 were provided by Sanofi-Aventis Research (Montpellier, France). Stock solutions of oxaliplatin (50 mM) and SR141716 (10 mM) were prepared in phosphate buffer saline (PBS) and dimethyl sulfoxide (DMSO), respectively and stored at -20˚C in the dark.

Cell culture conditions. Human colon adenocarcinoma-derived DLD-1 cells were obtained from the Interlab Cell Line Collection (IST, Genoa, Italy). Cells were routinely cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, in monolayer culture and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 in air. At confluence, the grown cells were harvested by means of trypsinization and serially subcultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich, Milan, Italy.

Drug treatment. In the experiments investigating the effect of oxaliplatin and SR141716 on cell proliferation, DLD-1 cells were seeded at a density of $2x10^5$ cells/5 ml of RPMI-1640 containing 10% FBS in 60-mm tissue culture dishes (Corning Costar Co., Milan, Italy). After 24 h, to allow for attachment, the medium was removed and RPMI-1640 containing the different concentrations of oxaliplatin and SR141716 was added to cells and maintained for 24 and 48 h.

A set of experiments was performed by incubating DLD-1 cells with increasing concentrations of oxaliplatin (from 0.1 to 10 μM) and SR141716 (from 0.1 to 10 μM).

In another set of experiments, DLD-1 cells were incubated with both oxaliplatin and SR141716. Specifically oxaliplatin, at the fixed concentration of 10 μM, was used in the presence of increasing concentrations of SR141716 (0.5, 1, 2, 5 and 10 μM). Similarly SR141716 at the concentration of 10 μM was used with the increasing concentrations of oxaliplatin (0.5, 1, 2, 5 and 10 μM). Each experiment included an untreated control and control with the equivalent concentration of PBS and DMSO used for adding oxaliplatin and SR141716, respectively. Triplicate cultures were set up for each concentration of used compounds and for controls; each experiment was repeated six times. Cell viability, determined using the trypan blue exclusion test, always >90%.

Assessment of cell proliferation. After DLD-1 cells had been cultured for 24 and 48 h with different concentrations of tested drugs, the proliferative response was estimated by colorimetric 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and [^{3}H]-thymidine incorporation in cell DNA.

To determine cell growth by colorimetric test, MTT stock solution (5 mg/ml in PBS, Sigma) was added to each dish at a volume of one-tenth the original culture volume and incubated for 4 h at 37˚C in humidified CO_2. At the end of the incubation period, the medium was removed and the blue formazan crystals were solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol).

MTT conversion to formazan by metabolically viable cells was monitored by spectrophotometer at an optical density of 570 nm. Each data point represents the average of three separate experiments with each experiment containing four wells.

^{3}H-thymidine proliferation assay. DLD-1 ($5x10^4$ cells/well) were cultured in triplicate in round-bottomed 96-well plates in a final volume of 200 μl of RPMI-1640, containing 10% FBS in 60-mm tissue culture dishes (Corning Costar, Cambridge, MA, USA). SR141716 and oxaliplatin were added to the cells at the concentrations ranging from 0.1 to 10 μM. The drugs were also added in combination, SR141716 was added at the fixed concentration of 2 μM and oxliplatin at 5, 2, 1 and 0.5 μM. After 48 h of incubation at 37˚C, cells were pulsed with 1 μCi of ^{3}H-thymidine (Amersham-Pharmacia Biotech, Cologno Monzese, Milan, Italy) and harvested after further 18 h of incubation. Radioactivity was measured in a scintillation counter (Wallac, Turku, Finland).

Drug combination analysis. The combination index (CI) was calculated by the CalcuSyn software based on the Chou-Talalay equation (21), which takes into account both potency (Dm or IC_{50}) and the shape of the dose-effect curve (22). Briefly, CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect and antagonism, respectively. Dose reduction index (DRI) representing the measure of how much the dose of each drug in a combination may be reduced at a given effect level compared with the doses of each drug alone.

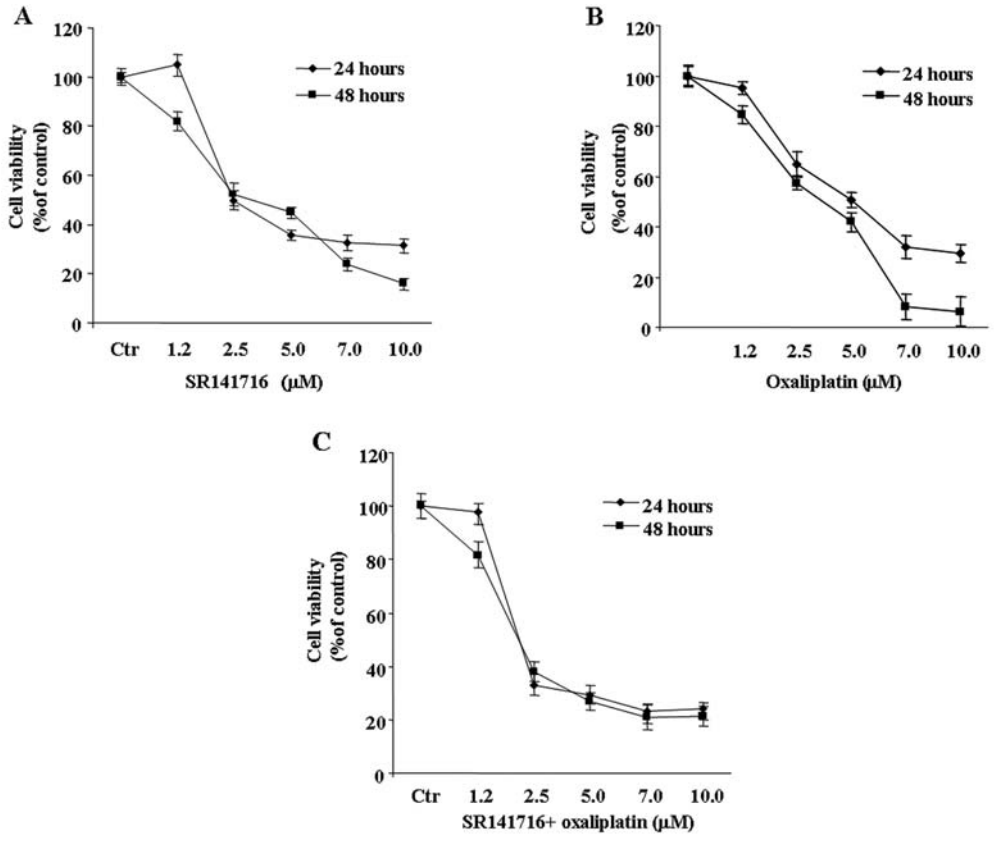

Figure 1. Anti-proliferative effect of SR141716 and oxaliplatin in DLD-1 cells assessed by MTT assay. Cells were seeded in 24-multiwell plates and drugs were added at the indicated concentrations. Cell growth assessment was done by MTT colorimetric assay after 24 or 48 h of treatment (see Materials and methods). Cell growth is expressed as percentage of control for each time point shown is the mean ± SD. A representative experiment (carried out at least three times) is shown.

Drug combination studies *in vitro* were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells vs. drug concentration after 24 or 48 h of treatment. To explore the relative contribution of each agent to the anti-proliferative effect, combinations with 1:1 oxaliplatin/SR141716 molar ratios, were tested. Assessment of drug interaction was performed calculating the Combinatorial Index (CI). CI/fractional effect curves represent the CI vs. the fraction of cells affected/killed by oxaliplatin and SR141716 in combination.

Statistical analyses. Analyses were performed using the Student's t-test. Results are expressed as means ± SD; P-values <0.05 were considered statistically significant.

Results

Effect of oxaliplatin and SR141716 on DLD-1 cell proliferation. In order to evaluate the drug effects on cell growth, DLD-1 cells where treated for 24 and 48 h with SR141716 (used concentrations ranging from 0.1 to 10 μM). The proliferation was evaluated with MTT proliferation assay. Results showed that SR141716 at the highest concentrations significantly inhibited DLD-1 proliferation. Inhibitory effects, similar to those induced by SR141716, were obtained with oxaliplatin (Fig. 1). The inhibitory effect was maintained with the combination of both drugs at equimolar concentrations with a similar effect at 24 and 48 h (Fig. 1).

SR141716 and oxaliplatin have synergic effect in inhibition of DLD-1 cell proliferation. In order to evaluate the combinatory effect of SR141716 and oxaliplatin, equimolar concentrations of both drugs ranging between 0.5 and 10 μM were analysed by combinatory studies. Drug combination studies *in vitro* were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells vs. drug concentration after 24 or 48 h of treatment. To explore the relative contribution of each agent to the synergism, combinations with different drug concentrations, at SR141716/oxaliplatin fixed molar ratios (1:1), were tested. Assessment of drug interaction was performed calculating CI. CI/fractional effect curves represent the CI vs. the fraction of cells affected/killed by SR141716 and oxaliplatin in combination (Fig. 2). The percentage of cells killed by drugs used in combination (fraction affected) and the dose reduction index (DRIs), which represent the order of magnitude (fold) of dose reduction obtained in combination setting compared with each drug alone, are shown in Table I.

^{3}H-thymidine incorporation assay: SR141716 inhibits DLD-1 cell line proliferation and increases the inhibitory effect of oxaliplatin. The effect of SR141716 was also investigated on DLD-1 cell line proliferation by ^{3}H-thymidine incorporation assay. Cells were cultured with SR141716 and oxaliplatin and combinations of both drugs at the concentrations indicated in Fig. 3 for 48 h, as at this time point the highest effect was observed by MTT assay. The combined effect of both

Table I. Fraction affected (FA) and dose reduction index (DRI) for rimonabant and oxaliplatin combination.

Concentration (μM)	FA	DRI		FA	DRI	
	SR + OXA 24 h	SR 24 h	OXA 24 h	SR + OXA 48 h	SR 48 h	OXA 48 h
1.25	0.1225	2.370	4.064	0.1532	7.738	0.992
2.50	0.2640	4.907	3.841	0.4573	25.50	3.043
5.00	0.3733	5.265	2.704	0.5155	16.97	2.004

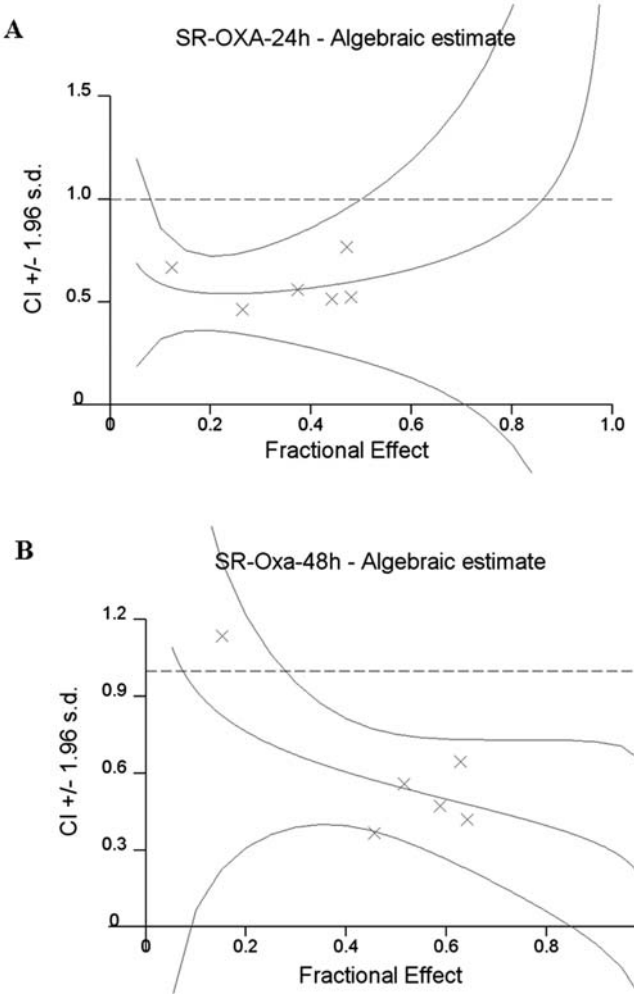

Figure 2. Combinatory effect of SR141716 and oxaliplatin in DLD-1 cell line. CI/fractional effect curves showed the CI vs. the fraction of cells affected by SR141716 and oxaliplatin in combination after 24 (A) or 48 h (B) of treatment. Combination analysis was done using the method described by Chou and Talalay (see Materials and methods). A representative experiment (carried out at least twice) is reported.

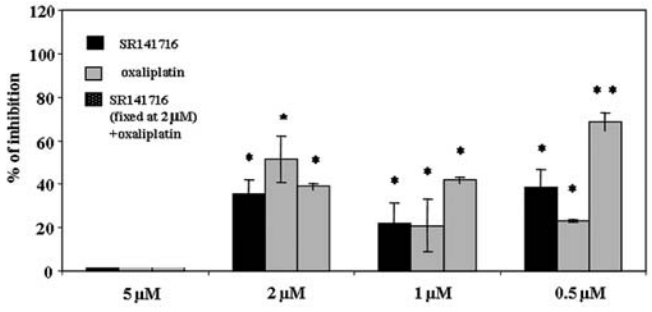

Figure 3. Antiproliferative effect of SR141716 and oxaliplatin in DLD-1 cells by ^{3}H-thymidine incorporation assay. Proliferation assay by ^{3}H-thymidine incorporation of DLD-1 titrated with SR141716, oxaliplatin and the combination of both drugs at the concentrations indicated in the graph. The mean percent of inhibition of SR141716 and oxaliplatin of three independent experiments is shown ± standard deviation (*P<0.05 calculated with respect to the control untreated cells, **P<0.05 calculated comparing the effect of SR141716 used in combination with oxaliplatin and substances used separately). The mean of the c.p.m. observed in three independent experiments is 41489.

SR141716 (2 μM) and oxaliplatin (0.5 μM) indicates that SR141716 increases the percent of block induced by oxaliplatin with respect to the effect exerted by the substances used separately at the same concentrations (Fig. 3).

Discussion

The anti-tumour activity of rimonabant has been recently suggested by numerous studies showing efficacy in thyroid (23), mantle cell lymphoma (24) and breast cancer (18). This study represents the first evidence showing anti-proliferative combinatory effect of rimonabant with oxaliplatin in human colon cancer cell line. We analysed the efficacy of rimonabant with respect to the currently used chemotherapic drug, oxaliplatin and performed combinatory studies to assess the relevant outcomes obtained by the simultaneous use of both drugs. MTT assays revealed in DLD-1 cells an anti-proliferative effect of rimonabant similar to oxaliplatin after 24 and 48 h of incubation, indeed the effect was maintained at equimolar combination of the drugs. In order to assess combinatory effects of synergism, summation, or antagonism of rimonabant with oxaliplatin, we analysed the combined effect of both drugs obtained by the MTT assay with Calcusyn program. We detected a synergistic effect in the inhibition of cancer cell proliferation obtained by the combination of SR141716 and oxaliplatin. As the separate substances produced a slightly higher effect after 48 h of incubation, we also assessed cell growth by ^{3}H-thymidine incorporation assay. The block of cell proliferation was confirmed, indeed SR141716 at the fixed concentration of 2 μM significantly increased the potency in cell proliferation inhibition of oxaliplatin at the low inhibitory dose of 0.5 μM with respect to the substances used separately. Previous findings reported the use of cytotoxic drugs in combination with other substances in the study of potentiation of anti-tumour effect. A synergic anti-proliferative effect has been previously observed in human colorectal cancer cell lines *in vitro* of oxaliplatin in combination with other substances, ZD6474

ZD1839 (Iressa) (20). Our findings could open a ıue for the use of SR141716, already reported as anti-tumour agent *in vitro* and in animal models (25), in combination with other currently used cytotoxic drugs in order to improve the anti-tumour treatment and also to potentiate the activity of existing chemotherapy agents. An additional advantage of these combinatory studies could be the possible reduction of the doses of chemotherapy drugs, thus limiting toxicity and side effects. In conclusion, this combination merits further investigations in both preclinical and clinical settings.

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