Immuno-modulatory Properties of a Quinolin-2-(1*H*)-on-3-carboxamide Derivative: Relevance in Multiple Sclerosis.

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Running title: Immuno-modulation of a quinolin-2-(1H)-on-3-carboxamide derivative.

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Abstract

Background: We have recently released the structure of a class of quinolin-2-(1*H*)-on-3-carboxamide derivatives and among them; the compound A2 has the highest CB2 receptor affinity and selectivity.

Objective: In this study we investigated the immuno-modulatory properties of A2 in lymphocytes isolated from peripheral blood of multiple sclerosis patients and healthy donors.

Methods: Cell proliferation was assessed by 3H-thymidine incorporation, cell viability and apoptosis by trypan blue, annexin V staining and western blot. Cell activation was investigated by flow cytometry and molecular pathways by western blot.

Results: A2 exerted anti-proliferative effects with down-regulation of TNF- α, IL-10 and Rantes in both cell types. No relevant changes were observed in cell viability between the two cell types. In cells form healthy subjects, A2 did not induce apoptosis, inhibited the cell cycle and similarly down-regulated in CD4+T the markers CD69, CD25, CD49d and CD54. Indeed, A2 also inhibited the phosphorylation of Akt, NF-kB, IKKα/β, ERK and blocked the expressions of Cox-2 and CB2 receptor. Differently, in cells from patients, A2 did not affect CD49d, while potently blocked CD54 expression. The inhibitory effects of A2 on Akt and Cox-2 expression were confirmed, whereas unchanged level of the CB2 receptor was observed in these cells.

Conclusion: We reported similar effects of A2 in both cell types; however, a different mechanism of action might be suggested in cell from patients concerning cell activation and CB2 receptor expression. Overall, these data suggest an anti-inflammatory profile of A2 with potential implication in multiple sclerosis.

Keywords: quinolin-2-(1*H*)-on-3-carboxamide derivative, lymphocytes, immuno-modulation, cell activation, CB2 receptor, multiple sclerosis.

Introduction

Recently, novel CB2 receptor agonists, 1,8-naphthyridine, pyridine and quinoline derivatives [1-5] have been designed and reported to be endowed of high CB2 receptor affinity and selectivity versus CB1 receptor in agreement with molecular modeling studies [1]. Some of these compounds exhibit pharmacological properties like inhibitory action on immunological human basophil activation mediated by the CB2 receptor [1,2]. We reported that these compounds exert immunomodulatory action with potential implication in multiple sclerosis (MS) and we suggested that higher CB2 receptor affinity is needed to reduce activation in lymphocytes isolated from MS patients [6]. Some compounds belonging to these classes of derivatives, have been shown to inhibit proliferation of several cancer cell lines. In particular, it was demonstrated that in DU-145 cell line, these ligands exert a CB2 receptor-mediated anti-proliferative action and decrease the CB2 receptor expression levels [5]. We also evaluated drug-permeability and oral bioavailability of 1,8-naphthyridine derivatives in assays on Caco-2 cells and on MDCKII-hMDR1 cells to estimate their blood brain barrier permeability. We showed that one of these compounds possesses a medium level of intestinal absorption and blood brain barrier permeability [7] whereas another compound showed high levels of intestinal absorption and blood brain barrier permeability (unpublished data).

More recently, we have also described the synthesis and pharmacological properties of new quinolin-2-(1*H*)-one- derivatives [8] characterized by 4-methylcyclohexylamido substituent in position 3 of the heterocyclic nucleus. This substituent has been selected on the basis of binding assays reported in our previous works for a series of 1,8-naphthyridin-2(1*H*)-on-3- carboxamides derivatives acting as potent and selective CB2 receptor ligands [2,4]. The new compounds have been tested on membranes prepared from HEK-293 cells expressing the human CB1 and CB2 receptors, to determine their affinities towards both subtypes of cannabinoid receptors. The binding affinities (Ki values) of the target compounds were evaluated by competitive radioligand displacement assays against the human recombinant CB1 and CB2 receptors over-expressed in HEK-293 and using [3H] CP-55,940 as the radioligand for both receptors [2]. In particular, the morpholinoethyl derivative, A2 was proved to be the compound with the highest affinity, with Ki values of 0.7 nM and 1.5 nM and 4.5 nM respectively. We have previously reported that analogs of the compound A2 dose dependently reduced cell viability on LNCaP, a prostatic cancer cell line expressing CB2 receptor. The same compounds showed immuno-regulatory effects suggesting their potential utility in MS [8]. In this study, we focused on A2 compound since among the quinoline compounds described up to date, A2 showed the highest CB2 receptor selectivity. In particular, given the relevant role of CB2 receptors and their agonists in pathologies like MS, in which this receptor has been proved to have neuro-protective role, we assess its effects in auto-reactive T cells isolated from MS patients to determine both inhibitory and anti-inflammatory properties comparing their action with that elicited by the some compound in cells isolated from healthy subjects.

Material and Methods

Drugs

Quinolin-2-(1H)-on-3-carboxamide derivative (chemical structure in Fig.1): A2 [8] was dissolved in dimethyl sulfoxide (DMSO). Vehicles were used as controls (not shown).

Isolation of human peripheral blood mononuclear cells (PBMC)

All donors gave written informed consent in accordance with the Declaration of Helsinki to the use of their residual buffy coats for research purposes. All MS patients were recruited at the University Hospital ‘‘Federico II’’ of Naples and gave written informed consent. All patients recruited were females (matched for sex with controls), at diagnosis. All the patients were naive to treatment and the expanded disability status scale (EDSS) was maximum 2. PBMC derived from buffy coats of healthy volunteers or from peripheral blood of MS patients were isolated as previously described [9]. 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 FBS (Sigma Chemical Co., St Louis, MO, USA).

Proliferation assays on human PBMC and cytokines/chemokines production.

PBMC isolated from ten MS patients and ten healthy donors ($2x10^5$ cells per well) were cultured in triplicate in round bottomed 96-well plates in a final volume of 200 ml of RPMI 10% FBS. Cells were stimulated with human myelin basic protein (MBP) (10 mg/ml) (Sigma). A2 was added to the cells at concentrations ranging from 0,3 to 10 μM. After 6 days of incubation, cells were pulsed with 1 μCi of 3H-thymidine (Amersham-Pharmacia Biotech, Cologno Monzese, Milano, Italy) and radioactivity was measured by scintillation counter (Wallac, Turku, Finland). Supernatants from cell cultures above described were harvested after 48 h to measure TNF-α, IL-10 and Rantes production at drug concentration of 10 μM. Assays were performed by Human Fluorokine Multianalyte profiling (MAP) Base Kit (R&D system).

Cell vitality assays

PBMC isolated from MS patients and healthy donors were cultured with MBP (10 mg/ml) in the presence and in the absence of the drug at 10 μM in RPMI 10% FBS for 6 days in 24-well plates. After the incubation, cells were stained with trypan blue and counted by mycroscopy.

Flow cytometry assays

To quantify cell cycle progression and apoptosis, cells ($1x10^6$cells) were cultured with MBP (10 μg/ml) and the drug at 10 μM in RPMI 10 % FBS for 6 days in 24-well plates. 10^5 cells from each experimental condition were stained with or annexin V FITC and/or propidium iodide (PI) to detect apoptosis and cell cycle, respectively and processed as previously described [10]. To analyze T cell activation markers cell were cultures as described above, 10^5 cells from each

experimental condition were stained with CD4-Cy-Chrome, CD54 (ICAM)-FITC, CD69-PE, or CD4-Cy-Chrome, CD25-APC and CD49d (VLA-4)-PE (BD–Bioscience) and incubated in the dark at 4 °C for 15 min as previously described [10] and analyzed by flow cytometry. The analysis of the activation markers was performed gating on the lymphocyte region of CD4+T cells and the expression of the markers in drug treated cells was compared to that of activated and not treated cells. Flow cytometric analysis was performed by Summit v4.3. (Dako) program.

Electrophoresis and immunoblots

Cell extracts derived from MBP-activated PBMC isolated from MS patients and healthy donors and treated with the compound at 10 µM for 6 days were processed as previously described [11]. Primary antibodies (1:1000) (host species: rabbit) specific for NF-κB p65 (Cell Signaling Technology Inc., Danvers, MA, USA) and its phosphorylated form, pNF-κB p65 (Cell signalling), IKKα/β (Cell signalling), pIKKα/β (Cell signalling), Akt (Cell signalling), pAkt (Cell signalling), Erk (Cell signalling), pErk (Cell signalling), Cox-2 and CB2 (Santa Cruz Biotechnology Inc.) were used. Immuno-detection of specific proteins was carried out with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Bio-Rad, Life Science Research, Hercules, CA, USA), by enhanced chemiluminescence system (Amersham GE Healthcare). Actin (Santa Cruz Biotechnology Inc., anti-rabbit) was used as control.

Statistical Analyses

Results were analyzed by Student's T-test; and are expressed as mean ± SD. P values <0.05 were considered statistically significant.

Results

Compound A2 inhibits cell proliferation, cytokine and chemokine production.

In our assays, we treated PBMC with MBP to activate our cells isolated from both healthy donors and MS patients. MBP-activated cells were treated with A2 at concentration ranging from 10 to 0,3 µM. Results obtained showed that in healthy donor cells there was a dose dependent inhibitory effect (Fig.2A) by A2, whereas in cells isolated from patients, inhibition of cell proliferation was detected only at the highest concentration (Fig.2B). Micromolar doses of synthetic CB2 ligands have been previously used in other studies suggesting their efficiency in the modulation of immune cell function [12]. To assess if the anti-proliferative effects of our compound could be associated to the down-regulation of cytokine and chemokine production, we measured the release of TNF-α, IL-10, Rantes in cell supernatants. At the highest concentration of 10 µM, we found that A2 was able to significantly decrease MBP-induced TNF-α, IL-10, Rantes secretion by PBMCs, both in MS patients (Fig. 2A) and healthy donors (Fig. 2B). Except for IL-10, the inhibitory effect of A2 was higher in cells isolated from healthy donor with respect to cells isolated from MS patients (Fig. 2A).

Compound A2 does not induce apoptosis.

In order to asses if the anti-proliferative effects of A2 might be due to cell death, we stained the cells with trypan blue. Data obtained showed that in cells from healthy donors, cell death was not detected whereas a modest inhbition of cell viability was observed in cells isolated from MS patients with respect to control MBP-activated cells However no singificant difference could be observed between both the cell types (Fig. 3). Since the inhibitory effect on cell proliferation was higher in healthy donor cells, we further explored in these cells a potential induction of apoptosis, in particular we stained the cells with annexinV/PI and observed that A2 treatment did not induce apoptosis (Fig. 4A). This result was confirmed by the expression of the pro-apoptotic marker Bax that was not induced following A2 treatment with respect to MBP-activated cells (Fig. 4B).

Compound A2 inhibits cell cycle progression.

As alternative explanation of the inhibition of cell proliferation, we analyzed the cell cycle progression in healthy donor lymphocytes following treatment with A2 at the concentration of 10 μM. We observed a marked reduction of the percentage of cells in the S phase of the cell cycle in drug-treated cells, whereas there were not relevant differences in the other phases of the cell cycle with respect to control MBP-activated cells (Fig. 5).

Compound A2 inhibits T cell activation markers.

In order to investigate if the anti-proliferative effects observed in PBMC might be associated to inhibition of cell activation, we analyzed the expression of specific T cell activation markers. In particular, we evaluated the expression of CD69, a molecule involved in lymphocyte proliferation, CD25, the IL-2 receptor alpha chain, the adhesion molecule CD54 (ICAM) and the integrin CD49d both involved in cell trafficking and lymphocyte migration. We observed that A2 inhibited the expression of CD69, CD25, CD54 (ICAM) and CD49d in a similar extent reaching about 40% of inhibition. In cells from MS patients, the effects were comparable to that of healthy donors for the activation marker CD69, the inhibition of CD25 was slightly higher and there was no inhibition of CD49d, whereas the expression of CD54 (ICAM) was almost totally inhibited (Fig. 6).

Compound A2 affects protein expression of NF-kB, IKK, Akt, Erk, Cox-2 and CB2 receptor.

Among the main molecular pathways involved in lymphocyte proliferation, we investigated the expression of NF-κB, the IKK complex that upon activation of its catalytic subunits frees NF-κB, the levels of Akt, a relevant protein of the phosphatidylinositol 3' –kinase (PI3K)-Akt signaling pathway that can phosphorylate a number of proteins including IkB that frees NF-kB and allows it to translocate to the nucleus to bind and subsequently activate target genes, thus regulating important immune cell functions. Furthermore, we analyzed the expression of MAPK

kinase by evaluation of its component ERK1/2 known to play relevant role in driving T cell proliferation and activation. Among the targets of NF-κB, we analyzed the expression of the enzyme Cox-2 following treatment with our drug. Finally, we also verify the effect of A2 on the levels of the CB2 receptor. We observed that MBP treatment increased the phosphorylation of the proteins above mentioned as well as the expression of Cox-2 and CB2 receptor with respect to un-stimulated cells (PBMC in the figure 8A, 8B). The effect of A2 in cells from healthy donors was a down-regulation of the phopshorylation of NF-κB, IKK, Akt, Erk, Cox-2 and CB2 receptor (Fig 8A) whereas in cells from patients the inhibition of pAkt and Cox-2 was maintained except that on the expression of the CB2 receptor that was unchanged with respect to the control MBP-activated cells (Fig. 8B).

Discussion

The utility of CB2 receptor agonists is emerging from recent studies suggesting their potential application in neurodegenerative diseases like MS. Indeed, in order to avoid the psycotropic effects associated to compounds activating the CB1 receptor, it has been pursued the development of novel classes of derivatives, 1,8-naphthyridine, pyridine and quinoline derivatives [1,2,5] endowed with higher and lower activity to the CB2 and CB1 receptors, respectively. In recent works, we demonstrated that the CB2 receptor affinity and selectivity was higher for a class of naphthyridine compounds, whereas it was lower for the quinoline derivatives [8] designed in the same study. Being the CB2 receptor primarily expressed in cells and organs of the immune system, the effects of such compounds have been evaluated in lymphocytes. In particular, the immuno-modulatory properties of these novel described CB2 receptor selective agonists, 1,8-naphthyridine, pyridine and quinoline derivatives [1,2,5] have been recently assessed [6]. It has been showed that the effects of compounds belonging to these three classes were similar in immune cells isolated from MS patients and healthy controls. In particular, the effects of compounds with different affinity for the CB2 receptors were compared. Although similar effects have been reported for the three classes of compounds, lower ability or no effect in decreasing cells activation was described for the pyridine compound, AF4. These results suggested that higher CB2 receptor affinity is needed to reduce activation in lymphocytes from MS patients. Recently, we reported the synthesis and pharmacological properties of a quinoline compound, A2 endowed with higher affinity and selectivity for the CB2 receptor with respect to other parent compounds of the same class [8]. In the present study, we analyzed the immuno-regulatory effect of this compound, A2 assessing differences between lymphocytes isolated from both MS patients and healthy donors. We found that the compound A2 was efficient to dose-dependently decrease cell proliferation in healthy donor cells, whereas the inhibitory effect in cells from MS patients was reported only at the highest concentration used. This difference in cell inhibition might be explained considering that cells isolated from MS patients are auto-reactive cells that are usually in a proliferative and activated state that might renders their inhibition more difficult with respect to cells isolated from healthy subjects.

Similarly, cytokine and chemokine production, TNF-α, Il-10 and Rantes, was inhibited by the compound A2. The down-regulation of cytokines and chemokines is suggested to be desirable in MS since they are commonly enhanced during the course of MS and interfering with their systems is considered an effective therapeutic approach in MS [13]. In particular, the down-regulation of TNF-α is not ascribable to cell death since we evaluated cell viability and induction of apoptosis following drug treatment. We observed that cells following treatment with our drug are mostly viable;the compound A2 does not affect cell viability in cells from healthy donors whereas slightly reduced it in cell from MS patients, however no significant differences were revealed between the two cell types. Furthermore, we assessed a potential induction of apoptosis in healthy donor cells. The results obtained showed that the compound A2 does not induce apoptosis as revealed by annexin V staining and confirmed by the expression of the pro-apoptotic marker Bax that was reduced by A2 treatment with respect to MBP-activated cells. In order to establish whether inhibition of proliferation might be due to arrest of the cell cycle, we examined in healthy donor cells the effect of the drug on cell cycle progression. The results obtained clearly show that our compound affects the cell cycle by reducing the percentage of cells in the S phase. The other phases of the cell cycle were not affected by the treatment with the substance. Since cell activation is a hallmark of autoimmune diseases like MS and adhesion molecules are critical players in the regulation of transmigration of blood leukocytes across the BBB in MS, we investigated the effects of test drug on specific T cell activation markers. We analyzed the effect of our compound on CD4+T cells, evaluating cell activation following treatment with the drug. In this case, we revealed differences for some activation markers in the drug effects between cells isolated from patients and those isolated from healthy donors. In particular, in healthy donor cells the inhibitory effect of A2 on specific cell activation markers, CD69, CD25, CD49d and CD54 (ICAM) was about 40% for all the markers used. Instead, in cells isolated from patients, we noticed that our drug was not able to reduce CD49d expression, whereas there was a potent inhibition of CD54 (ICAM) that was about the double of that observed in cells from healthy subjects. Previous studies also showed that the down-regulation of adhesion molecules by cannabinoid agonists interferes with the progression of MS [14], thus suggesting a therapeutic benefit derived by the use of modulators of these molecule involved in the regulation of transmigration of blood leukocytes across the blood–brain barrier in MS. The particular result observed on CD54 (ICAM) higher in PBMC from MS patients than in control cells, suggests a specific effect of this compound on this adhesion molecule in cells from patients. Indeed, it could be speculated that this compound with respect to other parent compound like pyridine derivative AF4, previously shown to be unable to reduce CD54 (ICAM) expression in MS patient derived cells, has a strong effect on this adhesion molecule suggesting that its particular structure is efficient and specific to inhibit CD54 (ICAM). It was previously showed that myelin activates FAK/Akt/NF-κB pathways and PI3K/Akt serves as upstream kinases for NF-κB activation by myelin [15]. In order to establish if our compounds could affect this signaling pathway, we analyzed the expression of Akt in MS patient and healthy donor derived cells. In MBP activated cells we found as expected, up-regulation of Akt

phosphorylation while following drug treatment, we observed down-regulation of the phosphorylation of Akt. The inhibitory effect on Akt phosphorylation was higher in cells isolated from healthy donors with respect to those isolated from patients. To further explore the effects on this signaling pathway we also demonstrated that NF-κB expression is affected by our compound, MBP activation induced NF-κB phosphorylation as consequence of IKKα/β kinase activation and our drug blocked both NF-κB and of IKKα/β phosphorylation in healthy donor cells. NF-κB is also a target of MAPK pathway, thus we examined the expression of Erk as component of this pathway. We observed that MBP activation induced Erk phosphorylation and our compound down-regulated it, thus suggesting that this drug is able to affect multiple signaling pathways. It is know that MS has a major inflammatory component that drives and orchestrates the disease. Our findings on NF-κB suggest that our compound can also affect inflammation. In order to further assess potential anti-inflammatory properties of our compound, we analyzed the expression of the enzyme Cox-2, target of NF-κB that is usually induced at site of inflammation and is a major player in inflammatory reaction in peripheral tissues. We found that A2 reduced Cox-2 expression in both MS patient and healthy donor derived cells. Finally, we assessed the expression of the CB2 receptor in PBMC isolated from MS patients and healthy donors. We observed that in healthy donor derived cells, MBP activation is able to induce the expression of the CB2 receptor protein with respect to inactivated PBMC, differently; in PBMC derived from MS patients, resting cells has a basal protein expression that is not modified by MBP stimulation, thus suggesting that in cells from patients this receptor is already up-regulated. Indeed, the treatment with our drug, while down-regulating the levels of the CB2 receptor in PBMC isolated from healthy subjects, does not affect the basal expression of the protein. These results suggest different mechanisms of action on the CB2 receptor of this drug in the two cell types, that should be further investigated to better understand the role of this drug and in general of this class of compounds in MS.

Conclusion

Our results show that the effects of quinolin-2-(1*H*)-on-3-carboxamide derivative A2 in lymphocytes isolated from MS patients and healthy donors suggest an anti-inflammatory profile of this compound, indeed, it has a specific effect on the adhesion molecule CD54 (ICAM). However, this drug does not affect the protein expression of the CB2 receptor in cells isolated from MS patients, differently from cell derived from healthy donors, thus suggesting a potential implication of this drug in MS.

Current and Future Development

The use of CB2 receptor agonists is recently emerging for their applicability in neurodegenerative diseases like MS. The design of novel compounds able to avoid the psycoactivity related to the CB1 receptor is a recent issue under investigation. The study in the same classes of derivatives that differ for chemical structure, activity to the CB2 receptor, provides a range of effects that allow not only to distinguish different basal effects between

different cell types like cells isolated from MS patients and those isolated from healthy donors, but also allow determining specific effects of a particular compound. In this case, the drug A2 elicits a potent inhibitory effect on CD54 (ICAM) that added to its anti-inflammatory profile suggests a potential application of this compound in MS. It is likely that also combinatory approaches might be adopted, as already done for Sativex that is a combination of tetrahydrocannabinol (THC) and cannabidiol (CBD) [16], using combination of drugs with different effects to obtain a better result to decrease for example symptoms, inflammatory state and improve the quality of life of patients.

Conflict of interest

The authors confirm that this article content has no conflict of interests.

Abbreviations

TNF-alpha: tumor necrosis factor-alpha

MS: multiple sclerosis

DMSO: dimethyl sulfoxide

PBMC: Peripheral blood mononuclear cells

EDSS: expanded disability status scale

MAP: Fluorokine Multianalyte profiling

MBP: myelin basic protein

PI: propidium iodide

PI3K: phosphatidylinositol 3' –kinase

NF-κB: nuclear factor-κB

Erk: extracellular-signal-regulated kinases

THC: tetrahydrocannabinol

CDB: cannabidiol

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Figure Legends

Figure 1. Structure of VL8

The chemical structure of VL8 is shown in the figure.

Figure 2. A2 derivative inhibits MBP-activated PBMC.

MBP-activated PBMC ($2x10^5$ cells per well) of healthy donors (A) and MS patients (B) were treated with the compound at the indicated concentrations, in triplicate, for 6 days. Proliferation was measured after 1 h of 3H-thymidine incorporation (1 mCi). The counts per minutes (c.p.m.) ± the standard deviation of the triplicates of a representative experiment out of five are shown (A). The activation following MBP stimulation was evaluated with respect to MBP untreated cells, (PBMC in the figure) ($^{\$}p<0,01$). The statistical significance was calculated with respect to the MBP-activated cells (*p,0,05, MBP in the figure). The table C represents the percent of inhibition ± standard deviation of cell proliferation calculated with respect to drug untreated and MBP activated cells isolated from MS patients and healthy donors (*p<0,01). The percent of inhibition reported is the mean of five independent experiments.

Figure 3. A2 inhibits cytokine and chemokine production.

The histograms represent the effects of A2 used at 10 μM, on TNF-α, IL-10 and Rantes production in supernatant collected after 48 h of culture of MBP-activated PBMCs isolated from healthy controls (A) and MS patients (B) (*p<0,01 calculated with respect to MBP-activated PBMC, MBP in the figures). The histograms reported show a single experiment that is representative of three independent experiments with reproducible results.

Figure 4. A2 effects on cell viability.

MBP- activated PBMCs derived from healthy donors and from MS patients were treated with the drug at the concentration of 10 μM and cultured for 6 days. After the incubation, cells were collected and stained with trypan blue. Cells were counted and the percent of cell vitality was evaluated on MBP- activated PBMC in the presence and in the absence of our compound. The histogram reported, shows the percent of live cells in MBP-activated PBMC and in the presence of A2. (p<0,01 calculated with respect to MBP-activated PBMC in MS patients)

Figure 5. A2 does not induce apoptosis.

MBP-activated PBMCs derived from healthy donors were treated with the drug at the concentration of 10 μM and cultured for 6 days. After the incubation, cells were collected and stained with Annexin V. Cells were analyzed by flow cytometry and the percent of apoptotic cells are reported for a single experiment representative of three with reproducible results (A). After the incubation with A2 cells were also processed to analyze Bax expression by western blot. A representative blot out of three is reported in histogram. The densitometric analysis reported is the mean of all the experiments performed (B).

Figure 6. A2 inhibits cell cycle progression.

In this figure the cell cycle progression of MBP activated-PBMC treated with the compound is showed. The flow cytometric profile is reported (A) along with the histogram on the right (B). The flow cytometric profile is representative of three independent experiments; we reported the percentage of cells in each phase of the cell cycle of a single experiment. The bars in the histogram (B) are calculated as mean of three independent experiments and represent on the y axe, the percent of cells in each phase of the cell cycle. The values of the histogram are reported in the table (C). The statistically significant decrease of the S phase (*p <0.01) following treatment with A2 is showed in the figure and is calculated with respect to MBP activated cells.

Figure 7. A2 inhibits T cell activation markers.

MBP-activated PBMC were treated with the drugs at the concentration of 10 μM and cultured for 6 days. After the incubation, cells were collected and stained with the antibodies indicated in the figure. Cells were analyzed by flow cytometry gating on the lymphocyte region of CD4+T cells. In the histogram, mean of at least four independent experiments, inhibitory effects of A2 on the expression of CD69, CD25, CD49d and CD54 in control PBMCs and patient derived PBMCs are showed. The control is represented by MBP-activated cells in which there is no

inhibition of these markers. The statistical analysis was performed on four independent experiments and the mean percent of marker inhibition ± the standard deviation of these experiments is represented in the histograms (*p<0,05 calculated with respect to MBP activated cells).

Figure 8. A2 controls Akt, IKKα/β, NF-κB, Erk, Cox-2 and CB2 receptor expression.

MBP-activated PBMC were treated with the compounds at the concentration of 10 μM and cultured for 6 days. After the incubation, cell extracts were prepared and protein expression was determined by western blot analysis. In the blots, the bands of un-stimulated cells (PBMC in the figure), MBP- activated cells (MBP in the figure) and the treatment with A2 are showed. The expression of phosphorylated Akt (pAkt) normalized on total Akt (Akt), Cox-2 and CB2 receptor normalized on actin is showed in control cells (A) and in patient derived cells (B). The densitometric analysis is also reported in the histograms for both MS patient and control derived cells. In addition, in control cells (A), the protein expression of pIKKα/β, pNF-κB, pErk normalized respectively on total IKKα/β, NF-kB, Erk is also showed along with their relative densitometric analysis. The blots are representative of four independent experiments and the densitometric analysis reports the mean of the values of all the experiments ± the standard deviation (*p<0,01significant inhibition calculated with respect to MBP-activate PBMC).