A Novel Highly Selective Cannabinoid CB2 Agonist Reduces in vitro Growth and TGF-beta Release of Human Glial Cell Tumors

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Abstract: Background: Cannabinoid receptors have been detected in human gliomas and cannabinoids have been proposed as novel drug candidates in the treatment of brain tumors.

Aims: To test the in vitro antitumor activity of COR167, a novel cannabinoid CB2-selective agonist displaying a high binding affinity for human CB2 receptors, on tumor cells isolated from human glioblastoma multiforme and anaplastic astrocytoma.

Methods: Glioma cell cultures were established from two glioblastoma multiforme and two anaplastic astrocytomas. Proliferation was measured in the presence or absence of COR167 with a bromodeoxyuridine (BrdU) cell proliferation ELISA assay. CB2 receptor expression was detected by western blotting. Apoptosis was assessed with phycoerythrin (PE) annexin V flow cytometry kit. TGF-beta 1 and 2 levels were analyzed in culture supernatants with commercial ELISAs.

Results: COR167 was found to significantly reduce the proliferation of both glioblastoma and anaplastic astrocytoma in a dose-dependent manner at lower doses than other known, less specific CB2 agonists. This activity is independent of apoptosis and is associated with a significant reduction of TGF-beta 1 and 2 levels in supernatants of glioma cell cultures.

Conclusion: These findings add to the role of cannabinoid CB2 receptor as a possible pharmacological target to counteract glial tumor growth and encourage further work to explore any other pharmacological effect of this novel CB2 agonist useful in the treatment of human gliomas.

Keywords: Glioma therapy, cannabinoids, CB2 receptor, TGF-beta, glioblastoma multiforme, anaplastic astrocytoma.

1. INTRODUCTION

Gliomas are brain tumors developing from glial cells with different histological phenotypes. Astrocytomas account for 25% of the diagnosed gliomas and are scaled from grade I to IV based on histological features [1]. According to the World Health Organization (WHO), grade I and II astrocytomas are classified as low-grade astrocytomas, while grade III and IV astrocytomas (referred to as Glioblastoma Multiforme [GBM]) are classified as high-grade astrocytomas. Patients with GBM have a median survival of 12 months after diagnosis and an average 5-years survival rate less than 5% [2, 3]. Despite extensive clinical and laboratory studies conducted on GBM, no effective treatments are still available for this disease [4]. However, based on fast growth of this tumor and its dependence on blood vessel formation for survival, an anti-angiogenic treatment strategy against GBM has been suggested [5]. In the last decade, both cannabinoid type-1(CB1) and cannabinoid type-2 (CB2) receptors have been detected in human cerebral tumors [6, 7]. These findings led to ascertain possible biological effects of cannabinoids on glioma cells in order to look for possible therapeutic molecules.

In experimental animal models, cannabinoid receptor agonists were found to inhibit glioma growth by modulating key signaling pathways resulting in apoptosis [8]. Moreover, CB agonists reduced tumor size by downregulating Vascular Endothelial Growth Factor (VEGF) [9], a factor correlating...
with the glioblastoma histological grade [10, 11]. Local administration of Δ⁹-tetrahydrocannabinol (THC), a natural CB1/CB2 agonist, in mice inoculated with a rat glioma cell line down-regulated the expression of Matrix Metalloproteinase (MMP)-2, an enzyme associated with a poor tumor prognosis [12]. However, selective activation of CB2 receptors reduced both malignant cell growth and angiogenesis in human skin tumor cells [13] as well as in rat glioma in vivo [14]. These findings support the CB2 receptor as a novel pharmacological target capable of counteracting glioma growth and progression. Nonetheless, although several CB2 receptor agonists with different affinities and selectivity have been synthesized [15, 16], none of these has yet been approved for the indication of brain tumor, due to several substantially unresolved issues, such as the low degree of receptor specificity and affinity, psychoactive side effects, and poorly acceptable dose-effect ratio.

In the last decade, COR167 (Fig. 1), a new cannabinoid agonist with high affinity and selectivity for the CB2 receptor has been synthesized [17] and found to display significant in vivo analgesic activity in a mouse pain model [18] as well as potent in vitro immunomodulatory effects on human immunocompetent cells, including down-regulation of proinflammatory cytokines, such as TGF-beta [19], at a dose as low as 10 nM never used for other CB2 agonists. To test whether this novel CB2 agonist could also display significant antitumor effects and to try to unravel the underlying mechanism, we evaluated the effect of COR167 on in vitro growth inhibition of glial cells derived from human gliomas with different histological grades and assessed apoptosis and release of TGF-beta from cell cultures.

Fig. (1). Chemical structure of COR167.

2. MATERIALS AND METHODS

2.1. Human Glioblastoma and Astrocytoma Cell Cultures

Primary human glioblastoma and anaplastic astrocytoma cells were isolated from glioma specimens obtained during neurosurgical operations, and preserved until their use under liquid nitrogen in cryopreservation medium [Dulbecco’s Modified Eagle Medium (DMEM)-20%, Fetal Calf Serum (FCS)-8%, Dymethylsulfoxide (DMSO)], as previously described [20]. Specimens from four different glial tumors were used. A list of the tumor characteristics is shown in the Supplementary Material (Suppl. Table 1S).

The microvessel fraction obtained with a previously described technique [21] was removed by centrifugation and primary glial cell cultures were implanted as previously reported [22]. Briefly, the glial cells were isolated from the middle fraction, obtained by density gradient centrifugation, and the pellet was resuspended in complete feed medium consisting of 20% heat-inactivated FCS, 5 mg/ml peptone, 25% glucose, 1% vitamins, 1% aminoacids, 1% antibiotic-antimyotic solution, and 2 mM 1-Glutamine in DMEM supplemented with 10% FCS, (all from Sigma Aldrich, Saint Louis, MO, USA) at 37 °C and 5% CO₂ humidified air, and the medium was changed twice per week. The cells grew quickly covering the entire dish surface in 1 week and, at this time, were trypsinized, counted and employed for further assays. Glial Fibrillary Acidic Protein (GFAP), a reliable marker of astroglial cells, was detected with immunofluorescence assay with an anti-GFAP monoclonal antibody (Exbio, Prague) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Calbiochem). This assay revealed 99% of GFAP-positive cells. No Factor VIII-positive cells were detected, indicating the absence of endothelial cells.

2.2. Cytotoxicity Assay

Compound (COR167) was synthesized at the Department of Biotechnology, Chemistry and Pharmacy of the University of Siena as previously described [17]. To test whether COR167 and the respective vehicle (0.1% DMSO in 0.9% DMEM) might cause cytotoxic effects on tumor cells, cytotoxicity assay was performed at different compound concentrations ranging from 10⁻⁹ M to 10⁻⁵ M. Cell viability (2.5 x 10⁵ cells/well) was assessed with a previously described 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazo-lium bromide (MTT) assay [23].

2.3. Tumor Cells Proliferation

The proliferation of glioblastoma and astrocytoma cultures in the presence or absence of COR167 in DMEM-20% FCS was assessed with a bromodeoxyuridine (BrdU) cell proliferation ELISA assay (Abcam Cambridge, UK), based on the BrdU incorporation into the DNA of dividing cells. For each glioma, quadruplicate cultures were analyzed. The assay was performed according to the manufacturer’s instructions. The intra-assay and inter-assay coefficient of variation was < 10%. Briefly, cells (2.5x10⁵ cells/well) were seeded in 96-well microplates (Nunc Cell Culture, Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 24, 48 and 72 h at 37 °C in the presence or absence of COR167 and, in some experiments, of the commercial CB2 antagonist AM630 (10 µg/ml, approximately 2x10⁻⁵ M) (Tocris Biosciences, Bristol, UK) added 15 min prior to addition of COR167 (10⁻⁵ M). In all assays a concentration curve of COR167 ranging 10⁻⁹ M to 10⁻⁵ M was used. This range was chosen as close to the CB2 receptor affinity values previously detected for COR167 [17]. BrdU was added to the cells for 24 hours, then the cells were fixed for 30 minutes, washed several times and then incubated with anti-BrdU monoclonal antibody for 1 hour at room temperature. After washing, the cells were incubated with peroxidase-conjugated goat anti-mouse antibody for 1 hour at room temperature and finally, after several washes, colour product developed after addition of Tetrathylbenzidine (TMB) solution and measured at ELISA photometer at 450 nm
wavelength. For each experiment, two independent assays were performed. The results were expressed as percent of inhibition, calculated according to the following formula:

\[ [100 - \text{(Absorbance COR167-treated glioma cells) / (Absorbance untreated glioma cells)}] \times 100 \]

2.4. Human TGF- beta 1 and 2 ELISA assay

To test whether the secretion of TGF-beta 1 and 2 can be induced or inhibited by COR167, we seeded tumoral cells (2.5x10^5 cells/well) in 96-well microplates (Nunc Cell Culture) and incubated for 24, 48 and 72 h at 37 °C with COR167 from 10^{-5} M to 10^{-8} M in the presence or absence of the commercial CB2 antagonist AM630 (10 µg/ml, 2x10^{-5} M) (Tocris Biosciences, Bristol, UK) added 15 min prior to addition of COR167 (10^{-5} M). Duplicate cultures were used for each experiment. At these times, the supernatants were collected and stored at -80 °C until use for TGF assay. For this purpose, we performed a commercial ELISA assay for TGF-beta 1 and 2, according to the manufacturer's instructions (Sigma Aldrich for TGF-beta 1 and Affymetrix, ThermoFisher Scientific, Waltham MA, USA for TGF-beta 2), using supernatants derived from glioblastoma and astroctoma cells untreated or treated with COR167. For both ELISAs, the intra-assay and inter-assay coefficient of variation was < 10%. For TGF-beta 1 ELISA, the supernatants were added in diluted, while for TGF-beta 2, the supernatants were diluted 1:50. In both cases, the colour product was developed after the addition of TMB solution and measured by spectrophotometer at 450 nm wavelength.

2.5. Immunoblotting Detection of CB2 Receptor

To test whether COR167 could modify CB2 receptor expression, western blot analysis was carried out. Membrane proteins were prepared from human brain astrocytoma and glioblastoma cultures in the presence or absence of COR167 and AM630 at the same concentrations as for MTT tests, according to a previously described method [24]. Briefly, the cells were lysed in 0.05 M Tris-HCl -10% aprotinin- 2mM Phenylmethylsulfonyl Fluoride (PMSF)- 2mM Ethylenediaminetetraacetic Acid (EDTA)- 1% nonyl phenoxypolyethoxyethanol NP-40 at pH 6.8, centrifuged at 12,000 rpm for 10 min and the proteins (40 µg/lane) were separated by 10% Tricine-SDS polyacrylamide gel electrophoresis [25]. The proteins were then electro-transferred to a nitrocellulose sheet by a Bio-Rad apparatus, renatured and incubated overnight with 3% bovine serum albumin-Tris Buffered Saline (TBS) pH 7.4 at 4 °C to block unspecific binding. The membrane was overlaid with mouse monoclonal antibody anti-human β-tubulin (2 µg/ml, Santa Cruz Biotechnology, CA, USA), for 2 hours at room temperature and, after washing with TBS-0.05% Tween 20, was then incubated with rabbit polyclonal antibody anti-human CB2 receptor (2 µg/ml, Thermo Scientific) overnight at 4 °C. After washing, the membrane was incubated with affinity-purified peroxidase-conjugated goat anti-mouse IgG antibody (1 µg/ml, Merck, Darmstadt, Germany) for 2 hours at room temperature and then with affinity-purified peroxidase-conjugated goat anti-rabbit IgG antibody (1 µg/ml, Merck) for 2 hours at room temperature. The colour reaction was developed with 0.075% 4-chloronaphthol in 0.05 M Tris-HCl pH 6.8 and 0.008% H₂O₂.

2.6. Apoptosis Analysis

To test whether COR167 induced apoptosis in human glioma cells, we used phycoerythrin (PE) Annexin V apoptotic staining flow cytometry kit (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Duplicate cultures were used for each assay. This assay measures the loss of membrane integrity accompanying the last stages of cell death resulting from either apoptotic or necrotic processes. After washing twice with cold Phosphate-Buffered Saline (PBS) pH 7.4, the cells, previously treated for 24 h at 37 °C with 10^{-5} M and 10^{-9} M COR167 in the presence or absence of 10^{-8} M and 10^{-9} M AM630, were resuspended in 1x binding buffer at a concentration of 10 x10^6 cells/ml. Then, 100 µl of this solution was transferred to a 5 ml culture tube and 5 µl of PE-Annexin V, as well as 5 µl of the vital dye 7-Amin compound (7-AAD), were added. After vortexing, the cells were incubated in dark for 15 minutes at room temperature. Finally, 300 µl of 1x binding buffer were added to each tube and the samples analyzed by flow cytometry. Data were acquired on a FACSCalibur® flow cytometer (BD Biosciences), 50 x 10^4 events were acquired in the gating window assessing an immunological gate around PE Annexin V (X-axis) cells and 7-AAD (Y-axis). Dot plots representing four parameters were created: Annexin-V+/7-AAD- cells and Annexin-V+/7-AAD+ cells. The ratio of apoptotic cells was then determined.

2.7. Statistical Analysis

Multiple comparisons were performed with ANOVA and Tukey post-test: \( P < 0.05 \) was considered significant. Data analysis was performed with PRISM, software version 7.2 (GraphPad, San Diego, CA, USA).

3. RESULTS

3.1. Cytotoxicity Assay

No significant cytotoxic effect of COR167 on both astrocytoma and glioma cells compared with respective untreated cells was observed at concentrations ranging from 10^{-9} M to 10^{-5} M after 24, 48 and 72 h of incubation, suggesting that the anti-proliferative effects of this CB2 agonist are not due to cytotoxicity on tumor cells (Fig. 2).

3.2. Glioma Cell Proliferation Assay

Exposure for 24 hours of the astrocytoma and glioblastoma cells to scalar doses of COR167 showed a very significant decrease (\( P < 0.0001 \)) in cell proliferation (by 49.5% to 51.7% and 50.3% to 53%, respectively) until 10^{-9} M and by 25.8% and 17.5%, respectively at 10^{-9} M. However, this anti-
proliferative effect was completely reversed ($P < 0.0001$) in the presence of the CB2 antagonist AM630 indicating that the inhibitory effect was due to binding of COR167 to CB2 receptor expressed on glioma cells. Similar results were observed after 48 and 72 h of incubation (Figs. 3A and 3B).

3.3. TGF-beta Levels in Cell Supernatants

TGF-beta 1 and beta 2 levels were found significantly reduced in cell cultures exposed to COR167 ($P < 0.0001$). TGF-beta 2 levels were higher than those of TGF-beta 1. There was no significant difference between TGF-beta 1 and 2 levels of
anaplastic astrocytomas compared to those of glioblastoma cells. This effect, which was completely reversed in the presence of the CB2 antagonist AM630 (Figs. 3C and 3D), has to be ascribed to CB2 receptor activation by COR167.

3.4. Expression of CB2 Receptor

The representative CB2 receptor expression pattern of two human gliomas was shown in Fig. (4). CB2 receptor was expressed in glioblastoma cells to the same extent as astrocytoma cells. When the cells were incubated with COR167, a significant reduction of the corresponding CB2 receptor band was observed ($P < 0.001$). However, the expression reduction was completely reversed by the CB2 antagonist AM630 ($P < 0.001$).

3.5. Cell Death/Apoptosis in Human Glioma Cells

To clarify the underlying mechanism of the anti-proliferative effect of COR167 on glioblastoma and astrocytoma cells, we assessed cell apoptosis by flow cytometry. A similar percentage of cells in early and advanced apoptosis was observed in both untreated glioma cells and cells exposed to COR167 without or with AM630 for 24 h (Fig. 5 and Table 1).

4. DISCUSSION

Our findings demonstrate that COR167, a novel highly specific synthetic CB2 agonist, is able to exert a powerful dose-dependent in vitro inhibitory effect on brain tumor growth at lower concentrations than those reported for other less selective CB2 agonists such as JWH133 (50 $\mu$g/day) in in vivo models (Fig. 5) [14]. This inhibitory effect is displayed on both glioblastoma and anaplastic astrocytoma cells with an activity extent still remarkable at 10 nM concentration. Cannabinoid agonists were found to display antitumor effects through a not yet completely cleared molecular pathway leading to apoptosis in glioma cells. THC, a natural cannabinoid agonist acting on both CB1 and CB2 receptors as well as JWH133, a synthetic CB2 agonist, reduced in vivo glioma growth in animal models triggering apoptosis in tumor cells [26-28]. It has been found that CB1 receptor is mainly localized in astroglial cells, whereas CB2 receptor is mainly expressed on microglial cells infiltrating human astrocytoma and thus representing the main CB2 receptor source in gliomas [6, 29]. Moreover, as the expression of CB2 receptors in human glioblastoma endothelial cells is increased compared to CB1 receptors, it has been speculated that CB2 receptors may play a crucial role in tumor growth through their activity on endothelial cells [30]. However, the CB2 receptor is further involved in the control of important neural cell processes, such as proliferation, differentiation, and survival [31]. An inverse relation between CB2 receptor expression and stage of cell differentiation was found in both glial cells and neurons suggesting that this receptor might act as a de-differentiating signal to induce a proliferative state [32].

The majority of these findings was obtained in established glioma cell lines presenting a more homogeneous morphological pattern. The in vivo morphological pattern of

![Fig. (4). CB2 receptor expression in glioblastoma and astrocytoma cells unstimulated or exposed to COR167. (A): Representative western blot of CB2 receptor expression; lanes 1 and 8: untreated glioblastoma and astrocytoma; 2-3-4-9-10-11: COR167 $10^{-5}$ M - $10^{-7}$ M - $10^{-8}$ M; 5-12; COR167 $10^{-5}$ M + AM630 $10^{-5}$ M; 6-13; COR167 $10^{-7}$ M + AM630 $10^{-5}$ M; 7-14; COR167 $10^{-8}$ M + AM630 $10^{-5}$ M. On the left, the molecular weight of proteins expressed in kDaltons (kD) is shown. (B): Expression ratios of CB2 receptor/tubulin obtained dividing CB2 receptor band densitometric values by those of tubulin. Due to the similar findings obtained in assays using AM630 at different concentrations, AM630 $10^{-8}$ M values only are shown. The values are expressed as mean ± SEM of respective bands in different blots. * $P < 0.0001$ (COR167 vs UNS and AM630) (ANOVA and Tukey post test).]
human gliomas is more complex often showing distinct morphological types within the same tumor. In our study, we used primary glial cultures prepared from human gliomas surgically removed from patients and thus more representative of the real clinical status. Moreover, the presence of 99% GFAP⁺ cells and the absence of Factor VIII-positive cells in these cultures limit the confounding role of other possible cellular components, such as endothelial cells expressing CB2 receptor, in assessing the real effects of CB2 agonist on glioma cells. In our study, the anti-proliferative effect of COR167 leads to CB2 receptor downregulation on glioblastoma cells. Upper left gate: dead cells; upper right gate: late apoptosis cells; lower left gate: viable cells; lower right gate: early apoptosis cells. 7-AAD = 7-aminoactinomycin D.

In the last two decades, TGF-beta has been found to play an important role in the development of many different tumor types, including gliomas, indicating TGF-beta pathway as an additional target for cancer therapy. In epithelial cancer, the role of TGF-beta was found “ambiguous” acting as a tumor suppressor in vitro but promoting in vivo transformation of benign skin tumors in carcinomas [36, 37]. However, malignant gliomas express TGF-beta ligands and receptors and several glioblastoma cell lines produce TGF-beta [38, 39]; in addition, increased levels of TGF-beta1 and TGF-beta2 were detected in the plasma of patients with glioblastoma [40]. Conversely, increased expression of TGF-beta1 was associated with reduced tumor growth in a human glioblastoma cell line in vivo making the role of TGF-beta controversial [41]. We showed that COR167 significantly reduces the levels of TGF-beta1 and 2 secreted by glioma cell cultures. This observation is consistent with our recent findings on the effects of COR167 on immunocompetent cells from multiple sclerosis patients, demonstrating a significant down-regulation of several proinflammatory cytokines including TGF-beta [19]. Whether reduction of secreted cannabinoid-induced cell death, such as the inhibition of lipoygenase (LOX)-enzyme [35]. All this data provides further demonstration of other and/or alternative intracellular targets that can be importantly modulated by cannabinoids contributing to their evident antitumoral effects.
TGF-beta levels may lead to reduction of proliferation of glioma cells remains to be elucidated, even though autocrine TGF-beta signaling was found to play a role in maintaining tumorigenicity of glioma–initiating cells, thus supporting a main role of TGF-beta in feeding glioma cell survival and proliferation [41].

CONCLUSION

The present study demonstrates that COR167, a highly specific cannabinoid CB2 receptor agonist, inhibits in vitro the growth of human glioma cells by a mechanism independent of apoptosis and linked to TGF-beta pathway that however remains to be fully elucidated. These findings add to the role of CB2 receptor as a novel pharmacological target in anti-cerebral tumor therapy and encourage further work to explore in vitro and in vivo models any other pharmacological property and effects of this selective CB2 agonist in the treatment of human gliomas.

LIST OF ABBREVIATIONS

7-AAD = Aminoadeninomycin D  
BrdU = Bromodeoxyuridine  
CB1 = Cannabinoid type-1  
CB2 = Cannabinoid type-2  
DMEM = Dulbecco’s Modified Eagle Medium  
DMSO = Dimethylsulfoxide  
EDTA = Ethylenediaminetetraacetic Acid  
FCS = Fetal Calf Serum  
FITC = Flourescein Isothiocyanate  
GBM = Glioblastoma Multiforme  
GFAP = Glial Fibrillary Acidic Protein  
IgG = Immunoglobulin G  
MMP-2 = Matrix Metalloproteinase-2  
MTT = 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium Bromide  
NP-40 = Nonyl Phenoxypolyethoxyethanol  
PBS = Phosphate Buffered Saline  
PE = Phycocerythrin  
PMSF = Phenylmethylsulfonyl Fluoride  
TBS = Tris Buffered Saline  
TGF = Transforming Growth Factor  
THC = Δ9-Tetrahydrocannabinol  
TMB = Tetramethylbenzidine  
VEGF = Vascular Endothelial Growth Factor

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of the University of Siena, Italy.

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research. All the humans used were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

All patients gave their informed consent to the use of tumor specimens obtained during neurosurgical operations.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher’s website along with the published article.

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