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The Anticancer Effect of Cannabinoid 2 Agonist L-759,633 on C6 and SH-SY5Y Cell Lines

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ABSTRACT:
Purpose: Previous studies have shown that cannabinoid 2 agonists have anticancer effects on different cancer cell lines. However, the effect of L-759,633 on the Neuroblastoma and glioma is still uncertain. The current study was intended to investigate the potential cytotoxic effects of a cannabinoid 2 agonist L-759,633 on the C6 and SH-SY5Y cell lines.
Material and Methods: In our study, the C6 and SH-SY5Y cell lines were used. For each cell line two cell groups were prepared to examine the effect of L-759,633 on C6 glioma and SH-SY5Y cell death. It was not applied any treatment to the cells in the control groups. Various concentrations (5, 10, 20, 40 and 80 µM) of L-759,633 was applied to the cells in the L-759,633 groups. After 24 hours the cell viability was examined using XTT assay. Total oxidant status (TOS) and Total antioxidant status (TAS) in the cells were measured by commercial kits.
Results: L-759,633 at the concentrations of 5, 10, 20, 40 and 80 µM significantly decreased the cell viability in C6 cells ($p<0.001$). It also significantly increased the TOS levels ($p<0.001$) whereas didn't alter the TAS levels ($p>0.05$). On the other hand this agent did not reduce SH-SY5Y cell viability.
Conclusion: L-759,633 has antiproliferative effect on C6 cells. Additionally, one of the potential mechanisms involved in this effect is activation the oxidant generation. However this drug didn't show antiproliferative effect on SH-SY5Y cell.
Keywords: *L-759,633, Oxidative Stress, cell viability, C6 Glioma, SH-SY5Y cell*

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INTRODUCTION

Glioblastoma multiforme (GBM) is a common category of malignant main brain tumors and one of the most hostile types of cancer. The survival after identification is usually merely six months to one year (Kleihues et al., 2002; Reardon & Wen, 2006). This is mostly because of the high invasiveness and reproduction rate of GBM. In addition, GBM shows high resistance to classic chemotherapy and radiotherapy (Maher et al., 2001). Current classic therapeutic strategies for the cure of GBM are merely palliative and include focal radiotherapy and surgical resection. A vast number of chemotherapeutic drugs have already been tested, yet no notable improvement on patient survival has been brought about (Guillermo et al., 2007). Neuroblastoma (NBL) is a common extracranial solid tumor in children. It is accountable for nearly 8% of childhood cancers and is characterized by changeable clinical behaviors demonstrating molecular diversities in the tumor (Maris et al., 2007). Medication for children with high-risk neuroblastoma includes chemotherapy, surgery, autologous stem-cell transplantation, immunotherapy, and radiation therapy. Despite the multimodality therapy, children with neuroblastoma have very faint outcomes, and the survivors encounter critical side effects related to medication toxicity (London et al., 2011). Therefore, the need for novel and less-toxic medical strategies to cure the

disease is urgent.

Modulation of the endocannabinoid system has been shown in different tumors. These changes contain the levels of generated endocannabinoids and the expression of their receptors. Modulation of the CB1 and CB2 expression has been shown to be associated with cancer cell motility, proliferation, invasion, and apoptosis (Kovalchuk & Kovalchuk, 2020). The anandamide and AEG levels are 2 - 3 fold higher in colorectal cancers and adenomas than in the healthy controls (Ligresti et al., 2003). Currently, increasing evidences propose that cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC), primary ingredients of cannabis sativa, and synthetic cannabinoids have anticancer activity (Galve-Roperh et al., 2000; Guillermo et al., 2012). Many cancer kinds (lymphoma, lung cancer, thyroid cancer, glioma, pancreatic cancer, skin cancer, uterine cancer, breast cancer and prostate carcinoma) have been referred to be responsive to the antiproliferative action of cannabinoids in a vast variety of experimental models which include cancer cell lines in culture and experimental animal models (Guillermo et al., 2012). The mechanisms implicated in the anticancer effects of cannabinoids comprise proliferation prevention, growth arrest (Galanti et al., 2008), initiation of apoptosis (Carracedo et al., 2006), activation of autophagy (Vara et al., 2011), angiogenesis suppression (Portella et al., 2003), and anti-metastatic effects (McAllister et al., 2011).

Recent information supports the presence of CB_2 receptors in the nervous system, particularly in microglial cells, glioma cells and astrocytes (Fernández-Ruiz et al., 2007).

Cannabinoid 1 receptors and cannabinoid 2 receptors were discovered in human cerebral cancers (Held-Feindt et al., 2006; Ellert-Miklaszewska et al., 2007). This discovery led to investigate possible biological efficacy of cannabinoids on glioma cells in order to try to find possible therapeutic agents. Cannabinoid receptor agonists were determined to suppress glioma enlargement by modulating key signaling routes resulting in apoptosis in experimental animal models (Carracedo et al., 2006). Furthermore, cannabinoid receptor agonists downregulated Vascular Endothelial Growth Factor (VEGF) which resulted in decreasing the tumor size (Blázquez et al., 2004). Until now, according to our knowledge, no published studies have demonstrated the possible efficacy of the CB_2 receptor agonist L-759,633 against neuroblastoma and malignant glioma. Therefore, the present study was conducted to investigate the possible antitumor efficacy of this agent on C6 and SH-SY5Y cell lines.

MATERIAL and METHODS

Cell Culture

Rat glioma C6 (CRL107) and human SH-SY5Y (CRL-2266) cells was obtained from American Type Culture Collection (ATCC). (6aR,10aR) – 3-(1,1-Dimethylheptyl)-6a,7,10,10a – tetrahydro – 1-methoxy-6,6,9 – trimethyl-6H – dibenzo[b,d]pyran (L-759,633) was acquired from Sigma-Aldrich (Cayman Chemical Company, USA). The cell lines were kept in Dulbecco's modified Eagle medium (DMEM; Lonza, Walkersville, MD, USA), that was mixed with 10 % (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich St. Louis, MO, USA) and 1 % penicillin/streptomycin (Gibco Thermo Fisher Scientific). The cells were planted in a 25 cm^2 flask and incubated at 37° in a 5 % CO_2 humidified atmosphere up to they reached about an 80-90 % confluence.

Cell Viability Assay

The viability of cells were evaluated using the XTT test (Roche Diagnostic, Germany). L-759,633 was dissolved in dimethyl sulfoxide (DMSO) and diluted in DMEM before administration. Cells were cultivated in 96-well plates at the density of 1×10^4 cells for each well in 100 µl of DMEM culture media and were permitted to stick, overnight. The following 24 hours, the increasing concentrations (5, 10, 20, 40 and 80 µM) of the drug was added to the two cell lines and the plates were then incubated for 24 hours. It was not applied any treatment to the cells in the control groups. After that, 50 µl XTT labeling solution was added to wells to find out the living cells, after that the plates were incubated at 37° for 4 hours. After mixing, the absorbance of each well was obtained using a microplate reader (Thermo, Germany) at 450 nm comparing to the control. The cell viability was stated in % linked to control (100 %

of viability) (Türe et al., 2020; Taskiran et al., 2021).

The Cells Homogenate Preparation

C6 cells were treated with L-759,633 at dose of 20 µM (median dose) for 24 hours. Using sterile tubes, cells from each group (treated group and untreated group) were brought together. They were centrifuged at two thousand turns a minute for about ten minutes. Then the supernatants were eliminated. The cells deposited on the underside of the tube were suspended by mean of PBS. Repeated freeze-thaw cycles were applied to the cells to burst and allow the internal components to escape. They were centrifuged at four thousand turns a minute for about ten minutes at a temperature of 4°C. Then, the supernatants were gathered for analysis of TOS, TAS and total protein levels (Taskiran, & Ergül, 2021).

Total Antioxidant Status And Total Oxidant Status Evaluation

Concentrations of TAS and TOS in cell supernatants were measured using an automated test method, which was developed by Erel (Erel, 2004a; Erel, 2005). The total antioxidant status assay depends on controlling the reaction proportion of free radicals by determining the absorbance of colored dianisidyl radicals throughout free radical reactions starting with the generation of hydroxyl radicals. Antioxidants in the samples should prohibit coloring proportional to their concentrations. The obtained results were expressed in µmol Trolox Eq/mg protein. In the total oxidant status, ferrous ions are oxidised to ferric ions when the medium contains enough oxidizers so that the test enables TOS levels to be determined by measuring the level of ferric ions through the use of orange xylenol. To calibrate the assay, hydrogen peroxide was used. The obtained results were expressed at µmol H_2O_2 Eq/mg protein. Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to determination of total protein levels in samples

Statistical Analysis

The statistical significance for the tests was found out using one way ANOVA followed by a Tukey post hoc test (SPSS 14.0 for Windows) for multiple comparisons between groups. Data acquired from the cell viability tests were stated as the mean ± standard error. Significance level was determined as $p<0.05$ and $P<0.001$.

RESULTS

Effect of L-759,633 on C6 and SH-SY5Y Cell Viability

In our study, increasing doses of L-759,633 (5, 10, 20, 40 and 80 µM) were examined on cell survival in both cell lines. As shown in figure 1, the tested doses of 5, 10, 20, 40 and 80 µM L-759,633 decreased the survival of C6 cells compared to the control group ($p < 0.001$; Figure 1). The IC_{50} was found to be 14.97 µM. However, L-759,633, in all of the doses, did not alter the survival of SH-SY5Y cells compared to the control group ($p > 0.05$; Figure 2).

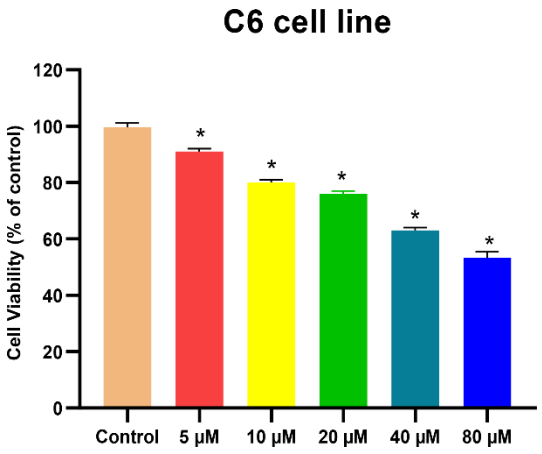

Figure 1. The effect of L-759,633 on cell viability in C6 cells. The data are given as mean ± SEM. *$p < 0.001$ as compared to the control group.

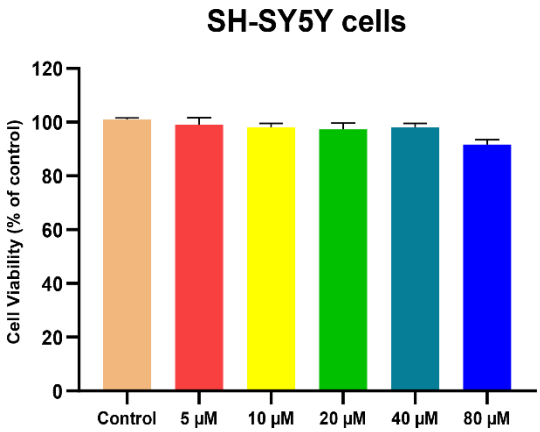

Figure 2. The effect of L-759,633 on cell viability in Human SH-SY5Y cells. The data are given as mean ± SEM. $p > 0.05$ as compared to the control group.

Effect of L-759,633 on TAS and TOS Levels in C6 Cell Line

The cells were treated with the single dose (20 µM) of L-759,633 for 24 hours. As shown in figure 3, the L-759,633 significantly increased TOS in C6 cells as compared to the control group ($p < 0.001$; Figure 3A). Besides, the L-759,633 didn't change TAS levels in C6 cells compared to the control group ($p > 0.05$; Figure 3B).

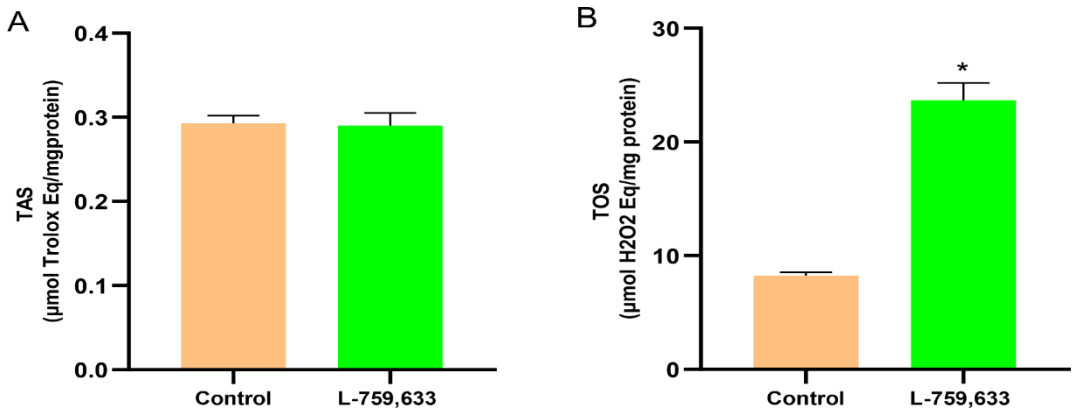

Figure 3. Effect of L-759,633 on TAS and TOS levels in C6 cells. The data are given as mean ± SEM. *$p < 0.001$ compared to the control group.

DISCUSSION

Even though there are diverse medical approaches, including chemotherapy, surgery, and radiation, the recurrence and resistance of neuroblastoma and malignant glioma frequently occur, requiring an alternative efficient medication of the diseases (Jacobsson et al., 2000; Suebsoonthron et al., 2017). It was essentially believed that except for endothelial and microglial cells, CB_2 receptors are unavailable in the central nervous system (CNS). In spite of that, the most recent studies indicated an expression of CB_2 mRNA and CB_2 immunostaining or immunoreactivity in various parts of the brain such as brainstem, basal ganglia, substantia nigra, cerebral cortex, hippocampus, and ventral tegmental area (VTA)(García et al., 2015; Den Boon et al., 2012; Lanciego et al., 2011; Onaivi et al., 2012; Van Sickle et al., 2005; Zhang et al., 2014; Schmöle et al., 2015). Since late 1990s, a large body of data has accumulated showing that different cannabinoids

produce antitumour effects in a wide range of experimental models of cancer (Velasco et al., 2016). The CB_2 receptor agonist CB13 induced a receptor expression and caused apoptosis in the cancerous cell line studied (Cianchi et al., 2008). Local application of Δ9-tetrahydrocannabinol (THC), a non selective cannabinoid agonist, in mice inoculated with a rat glioma cell line reduced the expression of the Matrix Metalloproteinase (MMP)-2, which is an enzyme correlated with a worse tumor prognosis(Blázquez et al., 2008). Selective activation of CB_2 receptors decreased both malignant cell enlargement and new blood vessels formation in human skin tumor cells (Casanova et al., 2003) as well as in the rat glioma in vivo (Sánchez et al., 2001). These data support the CB_2 receptor as a new pharmacological goal capable of suppressing glioma growth and progression (Cioni et al., 2019).

In the present study, the effects of different doses of cannabinoid 2 receptor agonist L-759,633 upon C6 and SH-SY5Y cells viability have been investigated. Selective cannabinoid 2 receptor agonist L-759,633 reduced the C6 cell viability. This reduction was dose- dependent, which aligns with what have been reported for the anticancer effects of selective CB_2 antagonists against glioma in previous studies. On the other hand, this agent did not reduce SH-SY5Y cell viability. This is in contrast to the study conducted by Wojcieszak et al. (2016) who demonstrated that CB_2 receptor agonist JWH-133 produced a concentration-dependent decline of SH-SY5Y cell viability and reproduction rate, and in contrast to the study conducted by Fisher et al. (2016) who showed that medication with cannabidiol (CBD) reduced the viability and invasiveness of NBL cells and stimulated apoptosis in vitro. In line with our study, Sezer et al. (2021) indicated that JWH-018, a non selective cannabinoid agonist, did not cause a significant reduction, in SH-SY5Y cell viability, did not modify apoptotic/necrotic rate, and did not induce genotoxicity in SH-SY5Y cells with one day exposure; Cioni et al. (2019) demonstrated that COR167 significantly decreased the proliferation of both anaplastic astrocytoma and glioblastoma in a dose-dependent manner; Sánchez et al. (2001) indicated that JWH-133 decreased the growth of tumors derived from C6 cell line, but this effect was prevented by the selective CB_2 antagonist SR144528.

The level of reactive oxygen species is one of the factors that play an significant role in the development and metastasis of tumors. In moderate levels, reactive oxygen species influence the tumor microenvironment by initiating metastasis and angiogenesis. On the other hand, the higher reactive oxygen-species-concentrations may induce a cancer cell apoptosis indicating that the reactive oxygen-species-levels critically determine tumorigenesis formations or apoptosis (Aggarwal et al., 2019; Ergul & Bakar-Ates, 2020). The total oxidant status (TOS) which is contained in diverse parameters that are used in the estimation of oxidative stress, is generally used to evaluate the overall oxidation state of samples (Erel, 2004b). In a like manner, the total antioxidant status (TAS) is used to estimate the overall antioxidant state of samples (Choi et al., 2015). In light of this knowledge, we estimated the effect of L-759,633 on TOS and TAS levels in C6 cells. Our results demonstrated that L-759,633 treatment significantly raised TOS levels in C6 cells supporting its cytotoxic effects. Yet, there was no significant alteration in TAS levels (Fig. 3). Rising TOS and not changing TAS levels, in reply to this, revealed that L-759,633 treatment clearly raises the oxidative stress in the treated C6 cells. In accordance with the relevant literature, the present findings showed that oxidative stress can be considered as a participator during cytotoxicity induced by L-759,633. However, our findings need to be confirmed by further studies which should investigate the mechanisms involved in L-759,633 anticancer activity.

CONCLUSION

The results of this study demonstrated that L-759,633, reduced the viability of C6 cells. These effects can occur, perhaps by inducing oxidative stress. Therefore, L-759,633 could have anticancer effects on the glioma cells. However this drug didn't show antiproliferative effect on SH-SY5Y cell. Further research is needed to address questions regarding possible mechanisms.

Conflict of Interests

The authors declare that they have no conflict of interest.

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