The antitumor activity of cannabidiol on lung cancer cell lines A549 and H1299: the role of apoptosis

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ABSTRACT

In the recent years, the application of new antitumor drugs has focused on the replacement of conventional chemotherapeutics with compounds derived from natural products. Cannabidiol (CBD) is one of the 113 cannabinoids derived from the plant Cannabis sativa and is characterized with complex and not entirely understood biological function. Unlike the other most abundant cannabinoid in Cannabis sativa – tetrahydrocannabinol, cannabidiol has low affinity to the endocannabinoid receptors and the manifestation of its activity does not appear to rely on the endocannabinoid system. Cannabidiol is used in the treatment of many diseases including some types of cancer. The aim of our study was to evaluate the cytotoxic activity of cannabidiol and its effect on the process of programmed cell death. This process is directly involved in the antitumor effect of many drugs. Two lung cancer cell lines, A549 expressing p53 and H1299-p53 negative, were used. Apoptosis was monitored by Anexin V assay and the activation of Caspase 3/7. We found that CBD treatment led to a dose-dependent apoptosis increase in p53 positive A549 cells. The level of apoptosis in p53 negative H1299 cells was much lower and did not seem to be dose dependent. However, the total cell viability was similar for the two cell lines, which was due to the increased necrosis observed in H1299 cells.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1,2]. Non-small cell lung cancer (NSCLC) represents 85% of all lung cancers and is characterized by genetic abnormalities, which lead to alterations in signalling pathways that are targets for drug therapies – as a result leading to drug resistance [3]. Therefore, the development of novel therapeutic alternatives for NSCLC patients is an area of intense investigation.

Cannabinoids have been used for many centuries to counter pain. The endocannabinoid system (ECS) is an evolutionarily conserved signalling pathway, which has diverse regulatory functions [4]. In the last two decades, a growing body of evidence had shown the role of ECS in a diverse scope of diseases, such as psychological disorders, motion disorders like Parkinson’s and Huntington’s disease, neuropathy, disseminated sclerosis, spinal cord trauma, atherosclerotic disease, heart attack, hemorrhagic stroke, high blood pressure, eye disease, abnormal weight and bone diseases. For each of these diseases the potential positive effect of cannabidiol (CBD), a good candidate for ECS modulator and/or regulator, has been demonstrated [4–9]. Several studies have demonstrated that cannabinoids also have antineoplastic effect and are usually accompanied with no negative side effects such as the ones produced by the conventional chemotherapy treatment [10,11].

Cannabidiol (CBD) exerts selective cytotoxicity and halts the proliferation of many types of cancer. These effects mainly rely on processes such as apoptosis and autophagy and they are observed in many types of cancer [12–16]. In in vitro experiments CBD showed the most powerful antineoplastic activity compared to other phytocannabinoids [11]. In in vitro experiments CBD has been established as a potent anti-metastatic agent in treatment of cervical cancer cells [17], alveolar and lung cancer cells [18,19] and breast carcinoma cells [20]. Furthermore, CBD has demonstrated antineoplastic effects on solid tumors in vivo [12,18,19].

The aim of our study was to evaluate the cytotoxic activity of cannabidiol and its effect on the process of programmed cell death – a process directly involved in the antitumor effect of many drugs. Two lung cancer cell lines, A549 expressing p53 and H1299-p53
negative, were used as a model of NSCLC. Apoptosis
was monitored by Anexin V assay and the activation
of Cas3/7. Caspase-3 (Cas-3) and caspase-7 (Cas-7) can
directly carry out apoptosis, which makes them great
targets for apoptosis monitoring [21].

Materials and methods

Cell cultures

A549 (p53 positive, ATCC, LGC STANDARDS) and H1299
(p53 negative, ATCC, LGC STANDARDS) lung cancer
cells plated in F-12K Medium (Thermo Fisher Scientific)
and RPMI-1640 Medium (Thermo Fisher Scientific)
respectively supplemented with 10% (v/v) fetal calf
serum (FCS, Thermo Fisher Scientific) and 1% penicillin/
streptomycin solution (Thermo Fisher Scientific) were
maintained at 37 °C in 5% CO 2 incubator.

Cannabidiol ethanol solution

CBD ethanol solution (10 mg/mL) was bought from
Sigma-Aldrich.

Etoposide

(Sigma-Aldrich) was used as a positive control in the
treatment of cells, as it is a chemotherapeutic with
known apoptotic action.

Cell viability assay

The cytotoxic activity of CBD was tested on a panel
of two human lung cancer cell lines using the MTT
assay as described previously [22]. Briefly, 2000 cells
were seeded into each well in 100 µL in octuplicates
in 96-well flat-bottom plates and incubated for 24 h
(37°C, 5% CO 2) to allow attachment of the cells. After
24 h the media were replaced with 100 µL of fresh
media containing the solution of the tested compound
in serial dilutions with concentration from 16 to
512 µmol/L. Cannabidiol was initially dissolved in 70%
ethanol and then diluted to the final concentration
with media. After 72 h of incubation, the medium was
changed with media containing MTT (Invitrogen) at a
final concentration of 0.5 mg/mL (a soluble tetrazolium
salt). The microplates were further incubated for 2 h
at 37°C and the formed formazan crystals were dis-
solved through addition of 100 µL dimethyl sulfoxide
(DMSO) into each well. The absorbance was measured
on an ELISA plate reader Varioscan Lux (Thermo Fisher
Scintific) at a test wavelength of 570 nm and a refer-
ence wavelength of 630 nm to obtain sample signal.

The statistical software GraphPad Prism v.7 was used
for data analysis.

Immunofluorescence microscopy

Cells of lung cancer cell lines H1299 and A549 were
seeded in numbers of 25 000 per well in a 24-well
plate (Corning Costar Flat Bottom Cell Culture Plate)
on glass coverslips previously sterilized with ethanol
and with ultraviolet (UV) light for 20 min each side.
After 24 h, the cells were treated with 10 µmol/L can-
babidiol solution and with etoposide at concentrations
of 200 µmol/L and 120 µmol/L, respectively, for H1299
and A549 cell lines. CellEvent Caspase-3/7 Green
Detection Reagent (Invitrogen, Thermo Fisher Scientific)
was used according to the manufacturer’s protocol for
detection of activated caspase 3/7 during apoptosis.
The coverslips were treated as described previously
[23], after 40 min the cells were fixed with 3.7% para-
formaldehyde in 1xPBS for 10 min at room temperature
(RT) and afterwards permeabilized with 0.1% Triton
X-100 in 1xPBS at RT for 2 × 5 min. The glass coverslips
were mounted in ProLong Diamond Antifade Mountant
(Thermo Fisher Scientific) containing 10 µg/mL DAPI
on microscope slides. The signals were obtained with filter
for the Alexa Fluor™ 488 dye on Zeiss AxioVert 200 M
microscope using a 63 × objective lens, equipped with
a CCD camera AxioCam MRm. The images were pro-
cessed with Image J.

Flow cytometry for apoptosis detection

For apoptosis detection a FITC Annexin V/Dead Cell
Apoptosis Kit (Thermo Fisher Scientific) was used [24].
Approximately 3 × 10 6 cells/mL treated with cannabidiol
concentrations corresponding to IC25, IC50 and IC75
values were stained in green with FITC Annexin V fol-
lowed by staining the DNA in red with propidium
iodide according to the manufacturer’s protocol. The
measurement was performed on a flow cytometer
FACSCalibur BD. The percentage of the cells at each
stage of apoptosis was determined by FlowJo v.9
software.

Statistical analysis

For statistical analysis, GraphPad Prism v.8 software
was used. Statistical differences in flow cytometry and
microscopic analyses were calculated by multiple com-
parisons function of one-way analysis of variance
(ANOVA). Differences were considered statistically sig-
nificant at P-values ≤ 0.05.
Results

Cell viability under cannabidiol exposure

Our foremost goals were focused on setting up a framework within which to determine the cytotoxicity of CBD in our cell cultures. To that end, we performed an MTT assay, which allowed us to establish the half-maximal inhibitory concentration (IC50) for CBD against the two cell lines after 72 h’ treatment. CBD showed cytotoxic effect for both cell lines. We performed this assay with the assumption that the IC50 values will give us basic information about the cytotoxicity of this compound, which is crucial in the development of more complex and informative experimental settings. The data we got from this experiment showed that the cells more resistant to the CBD treatment in in vitro conditions are H1299, which originated from a more aggressive form of lung cancer. These cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein. Thus, the lack of expression of the tumor suppressor gene probably necessitates the administration of higher doses of CBD to achieve cell death. Our results support the idea that CBD has the properties for more intricate research and the potential of becoming a powerful tool in the battle against lung cancer in particular, but also against many other types of cancer.

Assessment of cell apoptosis after cannabidiol treatment by flow cytometry

After establishing the 50% lethal dose of cannabidiol for both lung cancer lines, we check the level of apoptosis in both lines as the cause of cell mortality. Both lung cancer cell lines were treated with three different concentrations of cannabidiol, IC25, IC50 and IC75 (calculated from the cell viability curves, Figure 1) for 24 h. To measure the apoptotic cell number, we used a method which relies on the staining with Annexin V conjugated with FITC and a following DNA staining with propidium iodide. We performed this assay in order to verify the data that cannabidiol triggers apoptosis in lung cancer cells (Figure 2).

We observed that the percentage of apoptotic cells in the A549 cancer line was higher at all three applied CBD concentrations compared to H1299 (Table 1). Moreover, in A549 there was a significant increase in the number of cells in early and late apoptosis between treatments. In contrast, the percentage of necrotic cells in the H1299 cell line was significantly higher, especially at a higher concentration of the applied dose of CBD but the apoptotic cells were less. We determined that the number of cells in apoptosis increased in a dose-dependent manner, which suggests that apoptosis is the main cause of the cytotoxic effect that CBD has on A549 cancer cells. The difference in the response of both cancer cell lines, however, may be due to the fact that A549 is a cancer line with active p53 – one of the main player in apoptosis. This gives us reason to assume that cell apoptosis after treatment with CBD is regulated in a p53 dependent manner.

Immunofluorescent analysis of caspase 3/7 expression and localization

For immunofluorescent analysis we used CellEvent Caspase-3/7 reagent. Caspase 3 and caspase 7 share similar substrate specificity by recognizing the tetra-peptide motif Asp-x-x-Asp (D-x-x-D) [25]. Caspase-3/7 Green Detection Reagent [26] (Thermo

![Figure 1. Half-maximal inhibitory dose IC₅₀ values of cannabidiol treated cells.](image_url)

Note: The two lung cancer cell lines A549 and H1299 were treated with different concentrations of CBD in a range from 16 to 512 μmol/L for 72h exposure and were analyzed by MTT test. The data are normalized to the corresponding non-treated control cells. The mean values of three independent experiments performed in quadruplicates are presented with standard deviation (n=3) ±SD.
Fisher Scientific) is intrinsically non-fluorescent, as the DEVD peptide inhibits the ability of the dye to bind to DNA. However, after activation of caspase-3/7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response. We cultured cells on glass coverslips and treated them with IC25 concentration of CBD for 24 h. The concentration was chosen lower than the IC50, so that we can initiate a process of apoptosis without causing massive cell death. The localization of Cas3/7 was detected with a CellEvent® Caspase-3/7 kit and visualized under an epifluorescent microscope Zeiss AxioVert 200 M equipped with a CCD camera AxioCam MRm. For a positive control, we treated the cells with etoposide at previously established IC25 concentrations: 60 µmol/L and 100 µmol/L for A549 and H1299 respectively (Figure 3A and B).

We found Cas3/7 activation after treatment with etoposide or CBD in both cell lines, but apparently a stronger signal was observed in the A549-p53 positive cell line, compared to the H1299 cell line (Figure 3C and 3D). This result correlates with our results from flow cytometry, in which we also observed a stronger Anexin V signal in the p53 positive cancer cell line A549. It is interesting to note that the nuclei of cells treated with both CBD and etoposide appeared morphologically enlarged (swollen) compared to control untreated cells (Figure 3A and 3B).

**Table 1.** Percentage distribution of cells in early/late apoptosis and necrosis in A549 and H1299 cancer cell line.

|       | Control | IC25 | IC50 | IC75 |
|-------|---------|------|------|------|
| VC    | 97±0.1  | 79.9±6 | 65.6±5.8* | 60.9±9* |
| EA    | 2.4     | 3.80±1.8 | 5.98±3  | 5.05±1.7 |
| LA    | 1.3     | 7.48±4  | 10.6±2.6** | 14.5±3.8*** |
| NEC   | 0.07    | 8.86±5.2* | 17.8±4.6** | 19.6±6.6*** |

Note: Quantitative analysis of the percentage of viable cells (VC), early apoptotic (EA), late apoptotic (LA) and necrotic cells (NEC) from at least three independent experiments (n = 3) ± SD, show that CBD causes apoptosis in A549 and predominantly necrosis in H1299 in a time-dependent manner. The multiple comparisons function of one-way ANOVA was used to compare the mean of each column with the mean of a control column. Probability values were considered significant at the *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 levels.

**Figure 2.** Detection of apoptosis with FITC Annexin V/Dead Cell Apoptosis Kit - cells from the two cancer cell lines were treated with three different concentrations of CBD (IC25, IC50, IC75). Apoptotic cells were identified by the increase in the fluorescence intensity of FITC-labeled Annexin V and necrotic cells were detected by increase in the fluorescence intensity of PI. Representative scatter plots showing the distribution of Annexin V and PI staining for control cells and CBD treated cells. Cells are classified as “viable” (bottom left), “early apoptotic” (bottom right), “late apoptotic” (top right) or “necrotic” (top left).
Figure 3. Subcellular localization of cleaved, active caspase-3 in A549 (A) and H1299 (B) lung carcinoma cells. A549 and H1299 cells were cultured on glass chamber slides overnight at 37 °C. They were then analyzed using CellEvent® Caspase-3/7, a green detection cell permeable reagent for the intracellular distribution of cleaved caspase-3, under a fluorescence microscope. Green indicates cleaved caspase-3 and blue indicates nuclei. Quantification of the signal intensity of the active caspase-3/7 in control and cBD treated cells and corresponding ANOVA table (C and D). N is the number of cells that were scored for each tested group. Probability values were considered significant at the level of *p < 0.05, **p < 0.01, ***p < 0.005, **** p < 0.001; ns, non-significant.
Discussion

Every year around 1.8 million people are diagnosed with lung cancer. The high mortality rate which comes with this disease is often associated with the cancer cells becoming resistant to the commonly prescribed chemotherapy agents or the low selective cytotoxic capacity of these drugs. That is why, the search for new more selectively acting bio-derived medicines is on the rise.

In recent years, the medical and health-related applications of or CBD, has garnered tremendous attention. The most recent findings strongly support the further development of CBD as a promising anti-cancer drug. The mechanism of action of CBD and its potential applications in cancer therapy are the major focus of many authors [10].

In this study, we determined the toxicity of CBD on two lung cancer cells A549 and H1299. We found that the p53 positive cancer line A549 showed a lower IC50 value, probably due to the fact that H1299 cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein. In our previous studies, it was demonstrated that H1299 cells are less sensitive to the treatment with classical chemotherapeutic drugs such as cis-platin and Tamoxifen [22,27] as well; thus, the lack of expression of p53 is probably responsible for the observed higher drug resisters. It was interesting to examine the extent to which this cellular toxicity (mortality) is caused by the process of apoptosis. We performed an experiment which showed that indeed in A549 the percentage of apoptotic cells in early and late apoptosis was higher than in H1299. In contrast to the p53 positive cancer line, in H1299 the percentage of cells that were in the necrotic stage was higher. This required to assay the expression and localization of the Cas3/7 apoptotic markers in both cancer cell lines. Immunofluorescence analysis showed that in A549 (p53+) the expression of caspase 3/7 was much higher compared to H1299 cell line. The results also showed that the effect of CBD on lung cancer cell lines was dose dependent (Figure 2). In addition, the response of cancer cells depends on the presence of the tumor suppressor protein p53. However, not much has been reported on the induction of apoptosis via activation of p53 by Cannabis sativa-extracts.

The tumor suppressor protein p53 is one of the many substrates of caspase-3 [28,29]. p53 can direct a cell toward apoptosis by trans-activating a large number of pro-apoptotic genes and also this protein may induce programmed cell death by trans-activating the genes for some caspases themselves [29]. A good examples are the executioner caspase-6 and −7 which have been identified as transcriptional targets of p53 in cisplatin injury [30]. This is a probable explanation of the stronger signal observed in p53 positive cancer cell line A549 treated with CBD. An interesting perspective for future research is the in-depth study on the antitumor effect the CBD exerts on H1299 cells, which seem to be more resistant to the treatment. The level of apoptosis was twice to four times less depending on the dose in H1299 cells (Table 1); however the total cell viability was similar for the two cell lines. This is due to the increased necrosis observed in H1299 cells.

Conclusions

Having in mind the sheer volume of unknowns that come with these first data, we see many potential perspectives for future investigation of this phytocannabinoid. CBD could turn out a good tool in the fight with cancer. Despite the preliminary state of this study and the non-quantitive data it presents, it supports the idea that cannabidiol has the properties for more intricate research and the potential of becoming a powerful tool in the battle against lung cancer in particular, but also against many of the other types of cancer.

Data availability statement

All data that support the findings reported in this study are available from the corresponding author upon reasonable request.

Disclosure statement

No potential conflict of interest was reported by the authors.
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