Cannabidiol Affects Extracellular Vesicle Release, miR21 and miR126, and Reduces Prohibitin Protein in Glioblastoma Multiforme Cells[1],[2] (◉) CrossMark

Uchini S. Kosgodage[*],[3], Pinar Uysal-Onganer[†],[3], Amy MacLatchy[‡], Rhys Mould[‡], Alistair V. Nunn[‡], Geoffrey W. Guy[§], Igor Kraev[¶], Nicholas P. Chatterton[¶], E. Louise Thomas[‡], Jameel M. Inal[#], Jimmy D. Bell[‡] and Sigrun Lange[**]

[*]Cellular and Molecular Immunology Research Centre, School of Human Sciences, London Metropolitan University, London, UK; [†]Cancer Research Group, School of Life Sciences, University of Westminster, London, UK; [‡]Research Centre for Optimal Health, School of Life Sciences, University of Westminster, London, UK; [§]GW Research, Sovereign House, Vision Park, Cambridge, CB24 9BZ, UK; [¶]The Open University, Walton Hall, Milton Keynes, UK; [#]Extracellular Vesicle Research Unit and Biosciences Research Group, School of Life and Medical Sciences, University of Hertfordshire, College Lane, Hatfield, UK; [**]Tissue Architecture and Regeneration Research Group, School of Life Sciences, University of Westminster, London, UK

Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive form of primary malignant brain tumor in adults, with poor prognosis. Extracellular vesicles (EVs) are key-mediators for cellular communication through transfer of proteins and genetic material. Cancers, such as GBM, use EV release for drug-efflux, pro-oncogenic signaling, invasion and immunosuppression; thus the modulation of EV release and cargo is of considerable clinical relevance. As EV-inhibitors have been shown to increase sensitivity of cancer cells to chemotherapy, and we recently showed that cannabidiol (CBD) is such an EV-modulator, we investigated whether CBD affects EV profile in GBM cells in the presence and absence of temozolomide (TMZ). Compared to controls, CBD-treated cells released EVs containing lower levels of pro-oncogenic miR21 and increased levels of anti-oncogenic miR126; these effects were greater than with TMZ alone. In addition, prohibitin (PHB), a multifunctional protein with mitochondrial protective properties and chemoresistant functions, was reduced in GBM cells following 1 h CBD treatment. This data suggests that CBD may, via modulation of EVs and PHB, act as an adjunct to enhance treatment efficacy in GBM, supporting evidence for efficacy of cannabinoids in GBM.

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Introduction

Tumors that arise from glia or glial precursor cells are the most prevalent type of brain cancer and account for over 32% of all central nervous system (CNS) and approximately 80% of malignant primary CNS tumors [1]. Glioblastoma multiforme (GBM) is the most aggressive form and constitutes 50% of all gliomas and 15.6% of all primary brain tumors [2]. Long lasting and persistent headaches are the most common initial presenting symptom, often associated with seizures, visual disturbances, cognitive impairment and nausea and vomiting; the presentation depending on the location and growth rate

of the tumor. Effective treatment options remain very limited due to their aggressiveness and heterogeneity. Despite multimodal therapy consisting of surgery, radiation and chemotherapy, therefore only 28.4% of patients survive one year and 3.4% survive to year five [3]. This highlights the need for enhancing current therapeutic strategies with new approaches, including supplementary treatment with cannabinoids [4,5].

Extracellular vesicles (EVs) are lipid bilayer-enclosed structures, 30–1000 nm in diameter, which are released from parent cells and participate in cell-to-cell communication, both in physiological and pathophysiological processes, via transport of a variety of biological molecules. EVs participate in cell migration, differentiation and angiogenesis [6–11] and have been shown to play important roles in numerous pathologies including cancers [12–21]. Furthermore, the identity of circulating EVs and changes in their cargo may serve as reliable biomarkers of brain tumors and response to therapeutic treatment [22–25].

In GBM, EVs are emerging as key-mediators for intra/inter-tumor communication through horizontal transfer of functional proteins and nucleic acids, including mRNA, miRNA and lncRNA, through which GBM cells influence the microenvironment to promote tumor growth, angiogenesis, metabolism and invasion [26–29]. Both the regulation of EV biogenesis and changes in EV cargo are thus of great importance and drug-directed modulation of EVs is gaining increased interest for therapeutic use [27,30]. Novel ways for modulating EV release to limit tumor growth in vivo, and to sensitize various cancer cells to chemotherapy, have been highlighted by us and other groups [12,14,31–35].

Cannabidiol (CBD) is a phytocannabinoid derived from *Cannabis sativa* and known for its anti-neoplastic and chemo-preventive activities [36–38]. Known anti-cancerous effects of cannabinoids include inhibition of tumor proliferation, angiogenesis and induction of tumor cell death [5,37,39], while in GBM, additional effects on inhibition of invasiveness and stem-cell like properties have been observed [40,41]. The high resistance of GBM to standard therapy, consisting of surgical resection followed by radiotherapy in addition to concomitant and adjuvant chemotherapy with temozolomide (TMZ) [42], and the high recurrence rates of GBM tumors, is partly related to the presence of glioma stem-like cells [43]. A recent study showed that CBD enhanced radiation-induced death in GBM and also affected the stem/progenitor cells and astrocytes [44]. CBD has shown great promise in an exploratory Phase 2 placebo-controlled clinical study of a proprietary combination with tetrahydrocannabinol (THC) in combination with dose-intense TMZ in 21 patients with recurrent GBM (clinical trial NCT01812603) [45,46], while previously, CBD showed protective effects in murine models of glioblastoma [47,48]. CBD has also been shown to selectively inhibit GBM proliferation and to induce death of cultured human GBM cells [39], as well as being effective against other cancers [37].

We have recently shown that CBD is a novel modulator of EV release in several cancer cell lines [35] and we and other groups have shown that EV-modulators, including CBD, can significantly increase sensitivity of various cancer cells to chemotherapy [12,14,31–35]. Therefore, we set out to identify whether effects on EVs could be a hitherto overlooked contributing factor to the beneficial effects observed for CBD in GBM treatment. Besides modulating EV release, changes in EV cargo would also be of high importance and have for example been shown to change in GBM in response to TMZ treatment [49]. Thus we also sought to further establish whether CBD affected pro- and anti-oncogenic microRNAs (miRs) exported via EVs from GBM cells. The effect of CBD treatment was assessed on the main pro-oncogenic miR21, which is an anti-apoptotic factor in GBM, affects viability, senescence and invasion in GBM and is also enriched in EVs shed from GBM [50,51]. As an example of an anti-oncogenic micro RNA, effects on miR126 were assessed, as in GBM-derived patient samples, miR126 is significantly lower than in paired non-tumoural controls and related to high histopathological grades, but found to be elevated in GBM patients with better prognosis [52].

Recent studies have underpinned multifaceted roles of prohibitin (PHB) in cell metabolism, apoptosis, senescence, cell survival and immunity, and thus, cancer. PHB is also critical for mitochondrial function and integrity [53,54], while mitochondria are central to cancer survival and progression, in particular due to their central role in calcium signal control, which is altered in cancer [55–57]. Critically, CBD has been shown to modulate mitochondrial function, and thus, calcium signaling [58–62]. Importantly, increased PHB levels are linked to chemoresistance in cancers [63,64] and we have recently shown that CBD could lower PHB levels in three cancer cell types [35].

Here, we provide evidence that CBD reduces PHB protein levels and changes EV-mediated export of microRNAs to an anti-oncogenic signature in GBM cells. CBD-mediated modulation of EV profile in GBM provides novel insight into how CBD may work in GBM and furthermore informs improved strategies for intervention in GBM treatment.

Materials and Methods

Cell Cultures

LN18 (ATCC CRL-2610; grade IV glioblastoma derived from a male patient with a right temporal lobe glioma) and LN229 (ATCC CRL-2611; glioblastoma derived from a female patient with right frontal parietal-occipital glioblastoma), were cultured according to ATCC's recommendations at 80% confluence in 75 cm^2 flasks in complete Dulbecco's Modified Eagle's Medium (DMEM), with 5% foetal bovine serum (FBS) at 37 °C/5% CO_2. LN18 and LN229 are chemo-resistant and chemo-sensitive GBM cell lines respectively [65]; both cell lines are reported to form tumors in nude mice [66,67].

Cell Viability Assays

The viability of the cells was assessed after 1 h treatment with CBD (1 μM and 5 μM; GW Pharmaceuticals, U.K.), after 1 h treatment with TMZ (100, 200, 400 or 800 μM; Sigma, U.K.) and after 1 h treatment of CBD (5 μM) combined with TMZ (800 μM), compared to DMSO control treated cells. Cell viability was assessed before the start of every experiment using the Guava ViaCount cell death assay (Guava Millipore) as previously described [35].

Effects of CBD Treatment on EV Release from GBM Cells

The effect of 1 h CBD (1 and 5 μM) treatment on EV release was compared to control DMSO-treated cells. LN18 and LN229 cells were seeded at a density of 5 x 10^5 cells *per* well, in triplicate, in the presence of culture medium (pre-warmed DMEM, supplemented with 10% FBS; Sigma-Aldrich, U.K.). The cell preparations were thereafter washed with pre-warmed PBS (EV-free), and resuspended in pre-warmed serum- and EV-free DMEM (which had been centrifuged at 70,000 *g* for 24 h and filtered through a 0.22 μm

membrane) and plated at 5 x 10^5 cells. CBD (1 or 5 µM) in 0.001% DMSO was incubated with the cells for 1 h at 37 °C/5% CO_2; DMSO treated cells were used as controls. The plates were briefly placed on ice, the supernatant collected from each well, cell debris was removed by centrifugation at 200 g for 5 min and thereafter EVs were isolated from the remaining supernatant as described in section 2.5.

Effects of TMZ and CBD-TMZ Treatment on EV Release from GBM Cells

LN18 and LN229 cells were cultured and prepared for EV isolation and quantification as described in section 2.3 and respectively treated for 1 h with 5 µM CBD alone as before, for 1 h with TMZ alone (800 µM as determined by the cell viability assay; see sections 2.2 and 3.1) or for 1 h with a combination of CBD (5 µM) and TMZ (800 µM). DMSO-treated cells were used as controls.

EV Isolation and Quantification by Nanoparticle Tracking Analysis (NTA)

Cell culture supernatants from the procedures in sections 2.3 and 2.4 were initially centrifuged at 4000 g for 1 h for removal of cell debris and the resulting supernatant thereafter spun at 100,000 g for 1 h at 4 °C. The resulting EV pellets were then resuspended in Dulbecco's PBS (DPBS), centrifuged again at 100,000 g for 1 h at 4 °C and thereafter resuspended in 100 µl sterile EV-free PBS. Nanoparticle tracking analysis (NTA) was carried out using the NS300 Nanosight (Nanosight Amesbury, U.K.), equipped with a 405 nm diode laser and a sCMOS camera. Samples were diluted 1:10 in sterile-filtered EV-free DPBS and the number of particles in the field of view was maintained in the range of 20–40 with a minimum concentration of samples at 5 x 10^7 particles/ml. Camera settings were according to the manufacturer's instructions (Malvern), four 90 sec videos per sample were recorded and the obtained replicate histograms were averaged. Each experiment was repeated three times.

miRNA Analysis in GBM Cells and Derived EVs

For assessment of microRNA cargo in GBM derived EVs, LN18 and LN229 cells were cultured to a 75% confluency in T75 flasks in DMEM/5% FBS. The cells were washed with EV-free Dulbecco PBS (DPBS) and thereafter fresh EV and serum free medium was added, containing CBD (5 µM), TMZ (800 µM) or a combination of TMZ (800 µM) and CBD (5 µM), and 0.001% DMSO for control treatment. After 1 h treatment, the cell medium was collected for EV isolation (carried out as described in section 2.5), while the cells were pelleted for further RNA isolation and microRNA analysis. RNA was extracted from treated and control-treated cells and their respective EVs, using Trizol (Sigma, U.K.) and RNA concentration and purity was measured using the NanoDrop Spectrophotometer at 260 nm and 280 nm absorbance. RNA was reverse-transcribed to cDNA using the qScript microRNA cDNA Synthesis Kit (Quantabio, U.K.) according to the manufacturer's instructions. The resulting cDNA was used to assess the expression of microRNAs miR21 and miR126, while U6-snRNA and has-let-7a-5p were used as a reference RNA for normalization of miR expression levels. The PerfeCTa SYBR Green SuperMix (Quantabio, U.K.) was used together with MystiCq microRNA qPCR primers for both miR21 (hsa-miR-21-5p) and mir126 (hsa-miR-126-5p), obtained from Sigma (U.K.). The sequences for U6-snRNA primers were U6 forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′, Hsa-let-7a-5p forward 5′-CCGAGCTGAGGTAGTAGGTTGTATA-3′ and reverse

5′-CGCTTCACGAATTTGCGTGTCAT-3′ for both. The following thermocycling conditions were used: denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 2 sec, 60 °C for 15 sec, and extension at 72 °C for 15 sec. The miR21 and miR126 expression levels were normalized to that of U6, using the ΔΔC method [68]. Each experiment was repeated three times.

Western Blotting Analysis

LN18 and LN229 cells, from the treatment groups described in section 2.6, were pelleted and protein extracted using RIPA+ buffer (Sigma, U.K.) containing 10% protease inhibitor complex (Sigma P8340), by gently homogenizing the cell pellet with regular intervals on ice for 2 h. Thereafter, the cell preparation was centrifuged at 16,000 g for 20 min at 4 °C and the supernatant collected. The same procedure was carried out for extracting protein from isolated EV pellets. Protein extracts were re-constituted in 2× Laemmli sample buffer containing 5% β-mercaptoethanol, boiled for 5 min at 100 °C before separation by SDS-PAGE, using 4–20% Mini-Protean TGX protein gels (BioRad, U.K.), followed by Western blotting analysis. Approximately 5 µg of protein was loaded per lane and even transfer to nitrocellulose membranes (0.45 µm, BioRad) was assessed using Ponceau S staining (Sigma). The membranes were blocked for 1 h at room temperature in 5% BSA (Sigma) in Tris buffered saline (TBS) with 0.001% Tween20 (TBS-T), followed by overnight treatment at 4 °C with the primary anti-PHB antibody (ab75771, Abcam; 1/2000 in TBS-T) for cell lysates, while for EVs the primary antibodies against the EV-specific markers CD63 (ab68418) and Flot-1 (ab41927) were used at 1/1000 in TBS-T. Thereafter, membranes were washed in TBS-T, incubated for 1 h at room temperature with an HRP-conjugated secondary anti-rabbit IgG antibody (BioRad, U. K.; 1/4000 in TBS-T), followed by washing in TBS-T and visualization using ECL (Amersham, U.K.) and the UVP BioDoc-ITTM System (U.K.). HRP-conjugated anti-beta-actin antibody (ab20272, Abcam, 1/5000 in TBS-T) was used as the internal loading control and densitometry analysis was carried out using ImageJ.

Statistical Analysis

The graphs and histograms were prepared, and statistical analysis was performed, using GraphPad Prism version 6 (GraphPad Software, San Diego, U.S.A.). One-way ANOVA was performed followed by Tukey's post-hoc analysis; significant differences were considered as $P ≤ .05$.

Results

Cell Viability of GBM Cells Following CBD and TMZ Treatment

Cell viability of LN229 cells was not significantly affected after 1 h treatment with CBD at the concentrations tested (1 and 5 µM), while LN18 cell viability was reduced by 13.6% ($P = .0043$) in the presence of 5 µM CBD, but was not significantly affected by 1 µM CBD treatment (Supplementary Figure 1, A and B). The two cell lines differed in sensitivity to TMZ, with LN18 showing a 15% decrease ($P = .0023$) and a 23% decrease ($P < .0001$) in cell viability after 1 h treatment with 400 and 800 µM TMZ respectively, while the LN229 cells showed a non-significant 5% decrease in cell viability after 1 h treatment with 800 µM TMZ (Supplementary Figure 1, C and D). For further assessment of EV release and combinatory treatment with CBD (5 µM), 800 µM TMZ was thus the chosen

working concentration. Cell viability for 1 h combinatory treatment with TMZ (800 μM) and CBD (5 μM) resulted in a 24.2% decrease in cell viability (P = .0002) in LN18 GBM cells, while a 10.9% decrease in cell viability (P = .0118) was observed in the LN229 cells (Supplementary Figure 1, E and F).

Effects of CBD on EV Release from GBM Cells

Both LN18 and LN229 cells released EVs between 20–500 nm, with the majority of EVs in the isolate being in the range of 20–200 nm (Figure 1, A and B and Supplementary Figure 2). The EVs were characterized by electron microscopy and verified to be positive for the EV-specific markers CD63 and Flotillin-1 (Figure 1). In LN18 cells, the modal peak size of EVs released in untreated cells was 83.8 nm, while after 1 h treatment with CBD (1 or 5 μM) the modal peak size of EVs was 35.1 nm and 40.6 nm respectively (Figure 1C). In the LN229 cells, the modal peak size of EVs in untreated cells was 111.2 nm, while the modal peak size of EVs released after 1 h treatment with CBD (1 or 5 μM) was 104 nm and 125.5 nm respectively, with no significant change in modal peak size compared to that of vesicles released from untreated control cells (Figure 1D).

After 1 h treatment with CBD (5 μM), LN18 cells showed a significant reduction in EV release (both exosomes and MVs; Figure 2, A–C), while in the LN229 cells the opposite was observed as CBD increased EV release compared to DMSO treated control cells (Figure

2, D–F). In the LN18 cells, a 29% reduction was seen in exosome (≤100 nm) release (P = .0066), a 24% reduction in smaller (101–200 nm) MVs (P < .0001) and a 56% reduction of larger (201–500) MVs (P < .0001) following CBD treatment, compared to control treated cells (Figure 2, A–C). In the LN229 cells, a 65% increase of exosomes (≤100 nm) (P = .0144), a 50% increase of smaller (101–200 nm) MVs (P = .0001) and a 57% increase of larger (201–500) MVs (P = .0140) was observed following CBD treatment (Figure 2, D–F).

Effects of 1 h TMZ and Combinatory CBD-TMZ Treatment on EV Release from GBM Cells

The two GBM cell lines showed varying responses in EV release after 1 h TMZ treatment, compared to DMSO treated controls (Figure 2). In LN18 cells (Figure 2, A–C), TMZ significantly reduced release of the smaller (101–200 nm) MV subset by 20% (P = .0003; Figure 2B) but did not significantly affect exosome (≤100 nm) release or release of the larger (201–500 nm) MV subset (Figure 2, A and C).

In LN229 cells (Figure 2, D–F), TMZ treatment significantly increased exosome (≤100 nm) release by 20% (P = .0060), MVs in the 101–200 nm size range by 60% (P = .0008) and MVs in the 201–500 nm size range by 98% (P = .0406).

The two GBM cell lines differed in EV release profiles after 1 h combinatory treatment with CBD (5 μM) and TMZ (800 μM). In
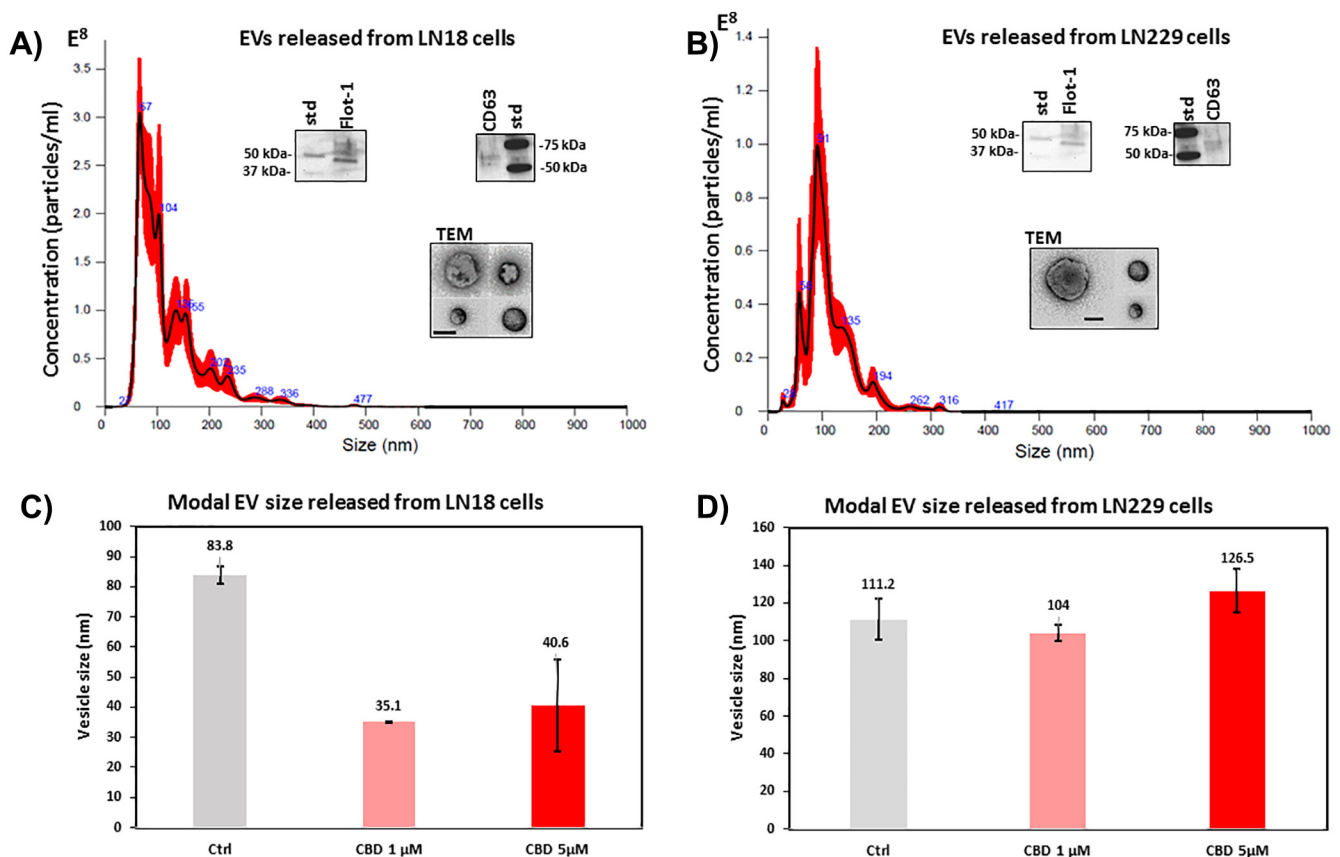

Figure 1. EV release in GBM cells under standard culture conditions and after 1 h CBD treatment. **A)** An NTA histogram, generated by Nanosight analysis, shows EVs released from LN18 cells under standard culture conditions; the size of EVs released falls mainly within the 25–300 nm range. EVs are verified to be positive for CD63 and Flot-1 and are also shown by TEM; the scale-bar is 100 nm. **B)** An NTA histogram of EVs released from LN229 cells, under standard culture conditions, shows an EV population of 25–300 nm. EVs are verified to be positive for CD63 and Flot-1 and are also shown by TEM; the scale bar is 100 nm. **C)** In the LN18 cell line, modal size of EVs released changed after 1 h CBD treatment. **D)** In the LN229 GBM cell line, modal size of EVs showed no significant change after 1 h CBD treatment.
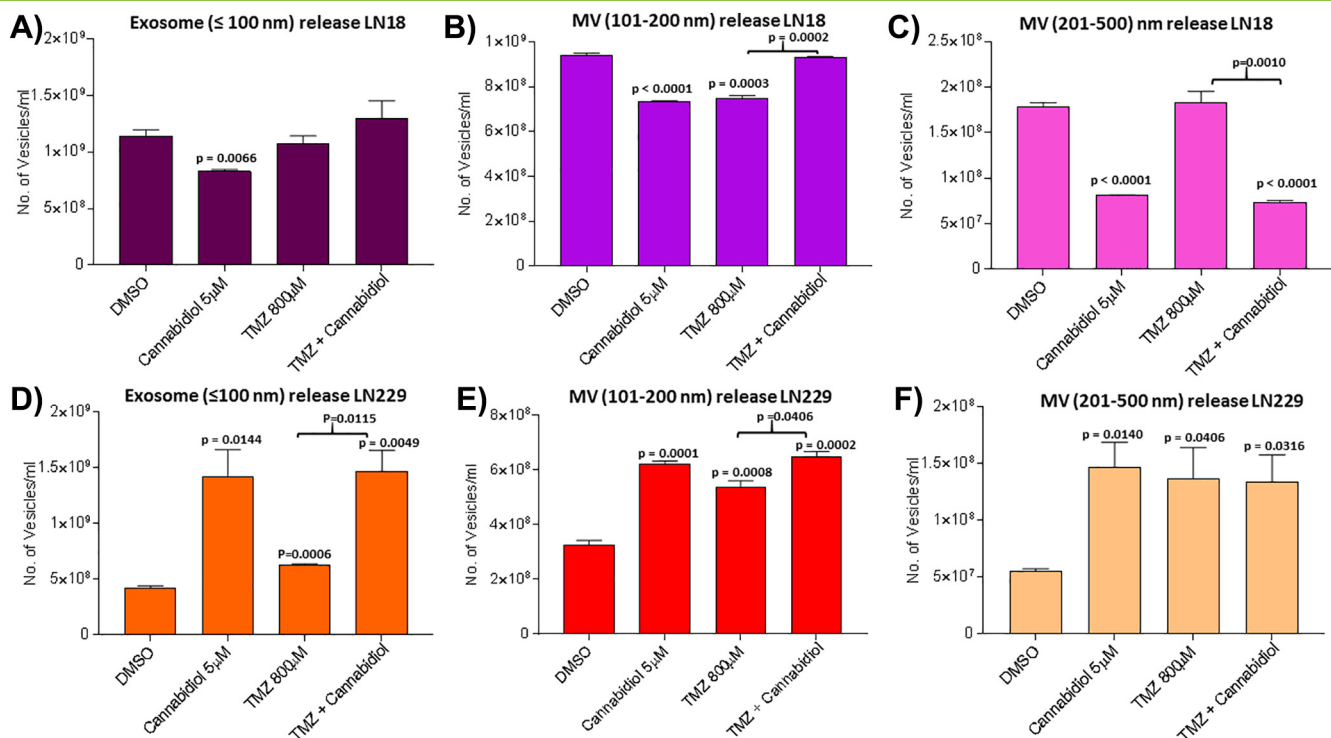
Figure 2. CBD and TMZ modulate EV release from GBM cells. EV release was assessed by NTA analysis after 1 h treatment with CBD (5 μM), TMZ (800 μM) and combinatory treatment of CBD (5 μM) with TMZ (800 μM). **A)** In LN18 cells, CBD significantly reduced exosome release. **B)** In LN18 cells, the release of smaller MVs was significantly reduced by CBD and TMZ but not after combinatory treatment, compared to DMSO controls. **C)** In LN18 cells, larger MVs were significantly reduced by CBD and CBD-TMZ combinatory treatment. **D)** In LN229 cells, exosome release was significantly increased in single CBD or TMZ treatment, as well as in the CBD-TMZ combinatory treatment, compared to control DMSO treated cells. **E)** In LN229 cells, the release of smaller MVs was increased in all treatment groups, compared to DMSO control-treated cells. **F)** In LN229 cells, the release of larger MVs was increased in all treatment groups, compared to DMSO controls. The P values indicated above the bars in the histograms represent significant changes compared to DMSO control; significant changes for TMZ versus combinatory treatment of TMZ with CBD are also indicated.

LN18 cells, CBD-TMZ combinatory treatment did not significantly affect release of exosomes (≤100 nm) or the smaller MV (101–200 nm) subset compared to DMSO controls, but significantly reduced release of the larger MV (201–500 nm) subset, compared to both DMSO control and TMZ treatment ($P \le .0001$ and $P = .001$ respectively; Figure 2C).

In LN229 cells, exosome release was significantly increased following CBD-TMZ combinatory treatment, both compared to DMSO controls and compared to TMZ alone ($P = .0049$ and $P = .0115$ respectively), but showed similar effects as CBD alone (Figure 2D); the same trend was observed for release of the smaller (101–200 nm) MV subset. The larger MV subset (201–500 nm) showed significantly increased release in all treatments, compared to DMSO control, while no significant difference was observed between the individual treatment groups (Figure 2F).

miRNA Analysis in GBM Cells and Derived EVs Following CBD Treatment

EVs isolated from LN18 and LN229 cells, and the respective cell lysates, were analyzed for changes in pro-oncogenic miR21 and anti-oncogenic miR126 following 1 h treatment with CBD (5 μM). Compared to DMSO treated controls, pro-oncogenic miR21 was significantly reduced both in EVs and the respective cell lysates after 1 h CBD treatment (Figure 3A). A stronger effect was observed for LN229-derived EVs, where miR21 in EVs was 5-fold reduced

compared to DMSO control ($P = .0001$; n = 3), while in LN18-derived EVs miR21 was 1.82-fold reduced compared to DMSO control ($P = .002$; n = 4; Figure 3A). In the respective cell lysates, a similar reduction of miR21 was observed in both GBM cell lines with an approximate 9-fold reduction ($P = .002$ for LN18; $P < .0001$ for LN229, respectively) compared to DMSO-treated cells. The relative levels of anti-oncogenic miR126 were significantly increased in EVs and the respective cell lysates after 1 h treatment with CBD (Figure 3B). In LN18-derived EVs a 2.5-fold increase ($P = .0005$; n = 4) in miR126 was observed, while in LN229-derived EVs a 6-fold increase was observed ($P = .003$; n = 3), compared to DMSO control treated cells. The same trend for increased miR126 was observed in the respective cell lysates with a 2-fold increase of miR126 in LN18 cell lysates ($P = .004$; n = 4) and a 6-fold increase in LN229 cell lysates ($P = .015$; n = 3), compared to DMSO treated controls (Figure 3B).

miRNA Analysis in GBM Cells and Derived EVs Following Combinatory TMZ-CBD Treatment

GBM cells were further assessed for modulation in microRNA cargo following 1 h treatment with TMZ (800 μM) alone, versus combinatory treatment of TMZ (800 μM) with CBD (5 μM). Pro-oncogenic miR21 was significantly reduced both in EVs released from LN18 and LN229 cells, as well as in the respective cell lysates, compared to TMZ treatment alone. Some differences were observed
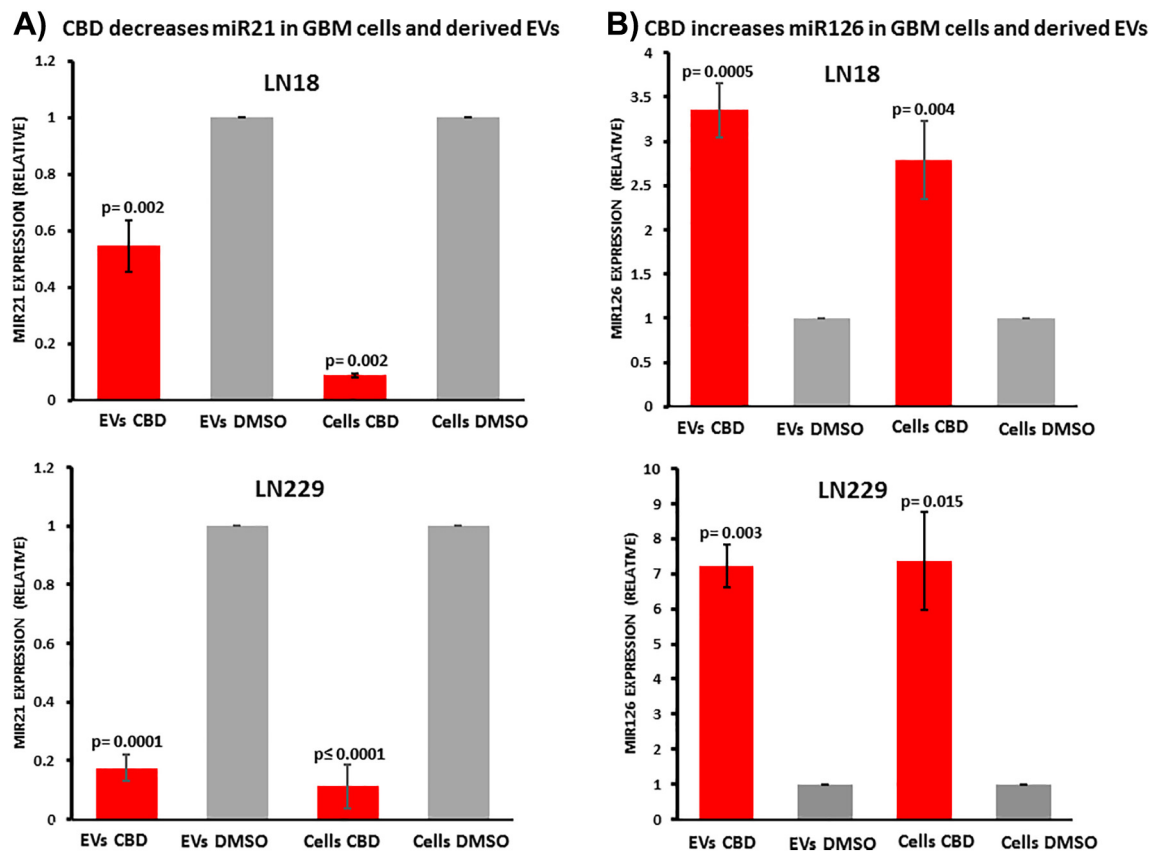
Figure 3. CBD reduces miR21 and increases miR126 in GBM cells and derived EVs. A) After 1 h CBD treatment (5 μM), pro-oncogenic miR21 was significantly reduced both in EVs released from LN18 and LN229 cells, as well as in the respective cell lysates, compared to DMSO treated controls. **B)** After 1 h CBD treatment, anti-oncogenic miR126 was significantly increased in EVs released from both LN18 and LN229 cells, as well as in the respective cell lysates, compared to DMSO controls. Exact P values for changes in relative miRNA expression are indicated (n = 4 for each treatment group for LN18; n = 3 for each treatment group for LN229). Data are normalized to U6-snRNA and has-let7a-5p.

between the two cell lines as follows: miR21 showed higher decrease in EVs released from LN18 (9.5-fold; $P < .0001$; n = 3) than LN229 (3.3-fold; $P = .0021$; n = 3), compared to TMZ treatment alone, while in the respective cell lysates, miR21 was 5-fold ($P < .0001$; n = 3) reduced in LN18 and 9.3-fold ($P < .0001$; n = 3) reduced in LN229 cells following CBD-TMZ treatment (Figure 4A). After 1 h CBD-TMZ treatment, anti-GBM associated miR126 was significantly increased in EVs released from both LN18 (3.84-fold; $P = .0018$; n = 3) and LN229 cells (3.25-fold; $P = .0032$; n = 3), compared to TMZ treatment alone. In CBD-TMZ treated cell lysates, miR126 was 11.84-fold ($P = .0015$; n = 3) increased in LN18 and 9.82-fold ($P = .0022$; n = 3) increased in LN229 cells, compared to TMZ treatment alone (Figure 4B).

Prohibitin Protein is Decreased in GBM Cells After 1 h CBD Treatment

In LN18 cells, a reduction of 11.3–37.7% in PHB protein levels was observed after 1 h treatment with CBD (5 μM), compared to DMSO treated controls (Figure 5A). A similar trend was observed in the LN229 cells, with PHB protein levels reduced by 15–15.7% after 1 h CBD treatment, compared to DMSO treated controls (Figure 5B). In LN18 cells, treated with a combination of CBD (5 μM) and TMZ (800 μM) for 1 h, PHB protein levels were reduced by 3–8%, compared to TMZ treatment alone (Figure 5C). The same

trend was observed in LN229 cells, with a 5–9% reduction in PHB protein levels following combinatory treatment (Figure 5D). For assessment of relative changes, band density of PHB was normalized against the internal β-actin loading control (Figure 5).

Discussion

While CBD has been found to be effective in anti-GBM treatment, and EVs have been related to GBM progression and invasiveness, no link has hitherto been made between CBD and EV release in GBM. We report here, for the first time, CBD-mediated changes in EV profile from GBM cells and furthermore show that microRNAs in CBD treated GBM cells, and their derived EVs, are modified to an anti-oncogenic signature. The modulation of miR21 has previously been shown to affect viability, senescence and invasion in GBM [51,69], while miR21 silencing was found to decrease tumor cell proliferation and tumor size, as well as enhancing apoptosis activation and improving animal survival in vivo [70]. In GBM samples, miR126 is significantly lower than in paired non-tumoural controls; patients with higher intra-tumoural miR126 levels have significantly improved survival duration than patients with lower miR126 levels [52]. In GBM tissues, average miR126 expression is found to be significantly decreased and relates to high histopathological grades, while over-expression of miR126 suppresses glioma cell proliferation and invasion in vitro, for example via the ERK pathway [71]. The
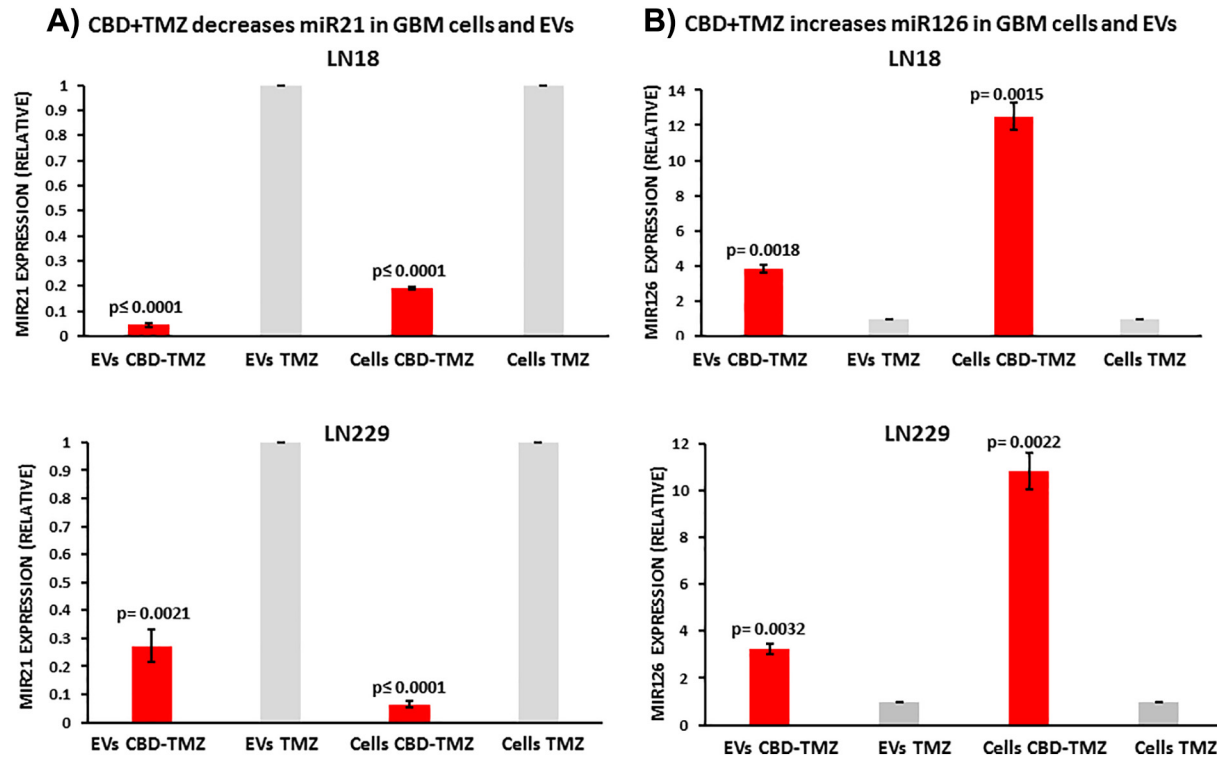
Figure 4. CBD in combination with TMZ more effectively reduces miR21 and increases miR126 export in EVs released from GBM cells, compared to TMZ alone. A) After 1 h CBD treatment (5 μM) in combination with TMZ (800 μM), pro-oncogenic miR21 was significantly reduced in EVs released from LN18 and LN229 GBM cells, as well as in the respective cell lysates, compared to TMZ treatment alone. **B)** After 1 h CBD-TMZ treatment, anti-oncogenic miR126 was significantly increased in EVs released from both LN18 and LN229 cells, as well as in the respective cell lysates, compared to TMZ treatment alone. Exact *P* values for changes in relative miRNA expression are indicated (n = 4 for each treatment group for LN18; n = 3 for each treatment group for LN229). Expression levels are normalized to U6-snRNA and has-let7a-5p.
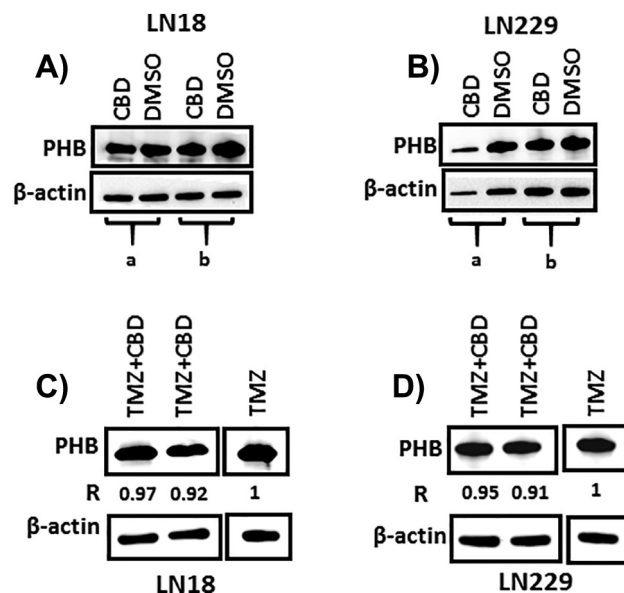

Figure 5. Prohibitin is reduced in GBM cells following CBD treatment. A) After 1 h treatment with CBD (5 μM), PHB protein levels were reduced in LN18 cells compared to DMSO treated controls; a = 11.3%, b = 37.7% reduction of PHB protein in CBD treated versus DMSO control treated cells. **B)** In LN229 GBM cells, reduced PHB protein levels were also observed after 1 h CBD treatment, compared to DMSO treated control cells; a = 15.7% and b = 15% reduction of PHB protein after CBD treatment respectively. **C)** Reduced PHB protein levels (3–8%) were also observed in LN18 cells treated for 1 h with CBD (5 μM) in combination with TMZ (800 μM), compared to TMZ treatment alone. **D)** In LN229 cells, reduced PHB protein levels (5–9%) were also observed in CBD-TMZ versus TMZ treatment alone. "R" indicates the change in PHB protein levels relative to β-actin, which was used as the internal loading control.

observed up-regulation of miR126 and increased EV-mediated export of miR126, found in the present study after 1 h treatment of GBM cells with CBD alone, as well as in combinatory application with TMZ, thus indicates an anti-GBM function via changes in this miRNA in response to CBD. In the same vein, reduced levels of miR21, after 1 h treatment with CBD, as well as in combinatory application with TMZ, indicates a strong anti-GBM activity via down-regulation of this miRNA and its extracellular transport via EVs. Notably, when applying a combination of CBD with TMZ in both GBM cell lines tested here, the relative-fold increase of miR126 and decrease of miR21 was more marked than after treatment with CBD or TMZ alone, thus indicating an enhanced anti-cancerous miRNA response when CBD is combined with TMZ. Interestingly, miR21 inhibition has previously been shown to enhance chemo-sensitivity of TMZ-resistant GBM cells in vitro [72].

PHB was found to be reduced in GBM cells following CBD treatment alone, and when combined with TMZ, compared to TMZ treatment alone. In a previous study, we showed that CBD-mediated changes in EV-release in three cancer cell lines associated with reduction in PHB levels, changes in mitochondrial function and sensitization to chemotherapy [35]. PHB plays multifaceted roles in cell survival immunity, metabolism, senescence and apoptosis [53,54]. The accumulation of PHB is a common cellular response to stress and has been shown to protect cancer cells from ER stress and chemotherapy-induced cell death [64]. PHB has previously been linked to GBM regulation [73,74] and shows dysregulated expression in gliomas [75–78]. PHB is associated with high grade gliomas [76] and the regulation of PHB, for example, via micro-RNAs, may be of pivotal importance in cancer treatment [74,79,80]. Interestingly, PHB accumulation occurs in mitochondria after chemotherapy treatment and *de-novo* accumulation has been shown to be associated with chemoresistance in melanoma in vitro, while knock-down of PHB sensitized melanoma cells to chemotherapy [64]. Changes in PHB levels have also been associated with melanoma cell proliferation, mitochondrial dysfunction, ER stress and melanoma cell apoptosis, in response to bornyl cis-4-Hydroxycinnamate from *Piper betle* stems [81].

Recently, TMZ has been shown to affect EVs released by GBM cells [49], and this relates to our findings here, where we detected TMZ-mediated modulatory effects on EV release and cargo composition in the two GBM cell lines. Higher levels of EV release in response to TMZ or CBD may imply a cellular response to aid drug efflux, but may also be indicative of a pseudo-apoptotic response, where apoptotic factors are still low enough for the cell to turn the apoptosome into EVs for export of hazardous agents [82,83].

Interestingly, after 1 h treatment with CBD, contrary to what was observed in LN18 cells, EV release from LN229 cells was significantly increased, and these EVs also carried 6-fold increased amounts of anti-GBM miR126 (compared to 3.5-fold increased amounts in LN18), as well as significantly reduced levels of pro-oncogenic miR21. Furthermore, combinatory treatment of CBD with TMZ resulted in many-fold increased levels of miR126 and reduced levels of miR21, compared to TMZ treatment alone. Thus, both CBD-mediated changes in EV sub-populations released and changes in EV cargo, alongside changes in PHB levels, may contribute to the known CBD-mediated sensitization of GBM cells to chemotherapy. The differences observed here between LN18 and LN229 GBM cells reflect the well-known complexity of glioblastomas [84]. At the same time, the identification of common pathways in the two GBM cell

lines tested here, may inform novel measures for treatment of this heterogeneous group of tumors. Recent findings during a placebo-controlled phase II clinical trial investigating CBD:THC in combination with dose-intense TMZ in GBM patients (clinical trial NCT01812603) has shown great promise, where the control group receiving TMZ had a 44% survival rate compared to the group receiving combinatory treatment of THC:CBD with TMZ, which had an 83% 1-year survival rate, and in addition showed median survival over 662 days, while the control group median survival was 369 days [45,46]. The CBD-mediated modulation of EV biogenesis, EV associated cargo and reduction of PHB levels, presented here, may thus be of great interest for refining application of CBD, in combination with standard therapy and chemotherapeutic agents, in anti-GBM therapy.

Conclusions

Here we show, for the first time, a modulatory effect of CBD on EV release and a CBD-mediated reduction in pro-oncogenic miR21 and elevation of anti-oncogenic miR126 in GBM cells. When used in combination with TMZ, CBD enhanced anti-oncogenic miR126 and reduced pro-oncogenic miR21 expression in GBM cells and GBM derived EVs, compared to TMZ treatment alone. Furthermore, we have also shown that PHB, a pleiotrophic protein involved in mitochondrial housekeeping, cell survival, immunity and chemore-sistance, was reduced in GBM cells upon CBD treatment. This supports emerging evidence that CBD has anti-cancer effects and indicates that CBD can be used to lower anti-chemotherapeutic responses to TMZ as well as modifying EV cargo to an anti-oncogenic signature in GBM.

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