



National Institute of Biology

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represented by Prof. Dr. Matjaž Kuntner, Director

subsequently called „**SERVICE PROVIDER**”

Date: 2.7.2019

Ljubljana, Slovenia

The responsible scientist: Prof. Dr. Tamara Lah Turnšek,

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represented by Mr. Roby Zoomer, Managing Director

subsequently called „**CONTRACTING ENTITY**”

***SUBJECT: The first Intermediate REPORT on work progress for the period from
1.3.2019- 30.6.2019***

PROJECT: DIAGNOSTIC PLATFORM FOR PRECISION CANNABINOIDS TREATMENT OF CANCER PATIENTS

The general aim of the Research project is to **develop formulations and to define the protocols for the treatment of high-grade brain tumours (glioblastoma-GB) with cannabinoids alone or as adjuvant therapeutics *in vitro* with the goal of *in vivo* translation to clinics.** Preclinical evidence supports high efficiency of extracts from natural occurring cannabinoids, such as THC and CBD) on inhibition of cancer glioblastoma cells' growth in invasion. Targeting GB cells by these cannabinoids may significantly improve standard disease treatment.

Specific aim of the first reporting period:

Service Provider will for the **Contracting Entity** set up methodology for testing the influence of natural cannabinoids on **cell viability** of the primary GB cells and GB cancer stem cells, derived from patients, and from the control, established GB cell lines

SUMMARY of the Report 1

I. Tumor Tissues

Patients selection and tumor sampling as well as patient's stratification regarding glioblastoma subtype is ongoing. Since March, we have **collected 10 patient samples** of which characteristics are in the process of being obtained from the Neurosurgery Department have been obtained (Table 1)

Genetic analyses on CB receptors and GB subtyping (coca 25 genes) has been performed, but the data are still analyzed by bioinformatics tools.

Protein analyses o CB1 and CB 2 receptors expression has been performed, showing variability the receptors expression in GB patients and their cells (see below) as was expected. Molecular markers of GB (histology, oncogenes) are analyzed at Pathology Department and will be available by next reporting period

II- Primary GB and GSC cell lines

are being established from GB tumors.

Cannabinoids CBD and THC significantly **reduce viability of U373 GB cells** an IC 50 values were determined in the control established cell lies, GSCs, NCH 644 and NCH 421k.

The results obtained with MGC **preparation ae in the range of the published data** by several other authors, as recently published.

At present **we cannot conclude** that the GSC cells are more susceptible for THC /CBD vs differentiated cells, possibly die to different culture conditions and this need to be farther explored

Dissemination

These Results were presented by Bernarda Majc and Prof. Tamara Lah Turnšek at the *Medical cannabis and cannabinoids* conference in Barcelona 2019, as a poster presentation entitled *Influence of cannabinoids on glioblastoma with differentially expressed receptors CB1 and CB2* (**Attachment 1**).

Experimental:

a) Patients selection and tumour sampling, as well as patient's stratification regarding tumour (GBM) subtype.

Since March 1st, we have received **10 patient tumour samples** (GBs) from the Department of Neurosurgery, Medical Centre Ljubljana (UKC) (**Table 1**). Neurosurgical and clinical/oncological data are being collected and recorded as part of another supporting project (**TRANSGLIOMA GLIOBANK-NIB**). Histopathological analyses of paraffin-embedded /frozen sections data are being performed at the Institute of Pathology, the Medical Faculty, University of Ljubljana.

Table 1: Clinical and histological parameters of glioblastoma patients.

Patient	Date of receiving the sample	Age+ (years)	Gender+	Survival * (months)	Diagnosis	Necrosis **	Angiogenesis**	Karnofsky ***	Subtype (MES, PN, CL)+§
1	5.03.2019				GB	yes	yes		
2	5.03.2019				GB	no	no		
3	8.04.2019				GB	no	yes		
4	15.04.2019				GB	no	yes		
5	23.04.2019				GB	yes	yes		
6	6.05.2019				GB	no	yes		
7	15.05.2019				GB	no	Yes		
8	5.06.2019				GB	no	yes		
9	5.06.2019				GB	Yes	yes		
10	7.06.2019				GB	no	yes		

Missing neurosurgical and clinical/oncological data and molecular analyses calculations are being collected.

+ Data will be obtained from UKC

++ Data acquisition/statistical are in progress (see c)

*Survival: from the date of the first operation till death, GB-glioblastoma

**Necrosis and angiogenesis, were analysed as yes or no in the laboratory before the processing the sample.

***Karnofsky score (at the time of first operation): patient functional impairment; 80-100: normal activity, able to work, no special care needed; 50-70: unable to work, able to live at home, varying amount of assistance needed; 0-40: unable to care for self, hospital care.

GB Subtype and cannabinoid receptors analyses of gene expression in all GB samples have been performed.

Experimental:

Genetic analyses of the GB tumours, that were snap frozen in liquid nitrogen and stored in the gene bank.

Control tissues: non-cancerous brain tissues and GB cells were also analysed. The gene expressions have been analysed by real-time PCR, using FLUIDIGM microfluidic technology.

Protein analyses of the GB tumours are being applied on paraffin embedded tumours by

Immunohistochemical (IHC) and on cells by immunocytochemical staining using Abcam monoclonal/or polyclonal antibodies

Results

I. Tumour tissue

Genetic analyses. All the samples are being:

Sub-typed as either mesenchymal or neural/proneural, based on expression of **12 characteristics genes**, as proneuronal (PN)/Neural (N) and mesenchymal (MES) markers or were of mixed phenotypes, as we published previously (see Breznik 2017).

Cannabinoid receptors: of **CNR1, CNR2, TRPV1 and GPR55** mRNA expression
Bioinformatic analyses of gene expression **are at present now being statistically processed** by quant
Genius software (**data not yet available**).

Protein analyses

We demonstrated that both CB1 and CB2 receptors are differentially expressed in GB tissue samples, (Figure 1)

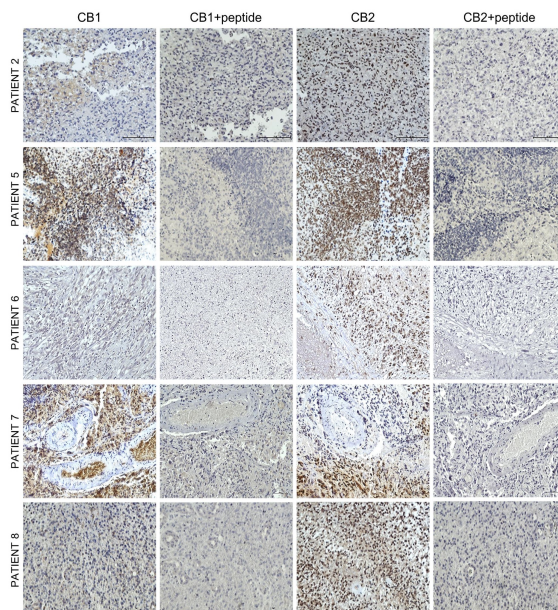


Figure 1 Figure 1: CB1 and CB2 are differentially expressed in GB tissue samples and GB cells. Immunohistochemical staining of CB1 and CB2 receptors (brown) in GB tissue samples. Cell nuclei were counter stained using haematoxylin (blue). Scale bar represents 50µm.

Table 1-IHC Summary;

Immunostaining intensity: 1=weak, 2=moderate, 3=strong;

% positive cells: 0%=0 / 1-33%=1 / 33-66%=2 / 66-100%=3

Overall score: intensity x% positive cells

Localization: m=membrane, n=nuclear, c=cytoplasmic

PATIENT	INTENSITY	LOCALIZATION	PERCENTAGE	SCORE
PATIENT 8	1	n/c	5	5
PATIENT 7	2	n/c	5	10
PATIENT 6	0	-	0	0
PATIENT 5	3	c	5	15
PATIENT 2	1	c	5	5
GB	1	c	5	5
	OVERALL	LOCALIZATION	PERCENTAGE	SCORE
		CB1	CB2	

II) GB cells and GB stem cells cytotoxicity/ viability inhibition by cannabinoids

a) Receptors protein analyses

We demonstrated that both CB1 and CB2 receptors are differentially expressed in GB and GSC cells (Figure 2)

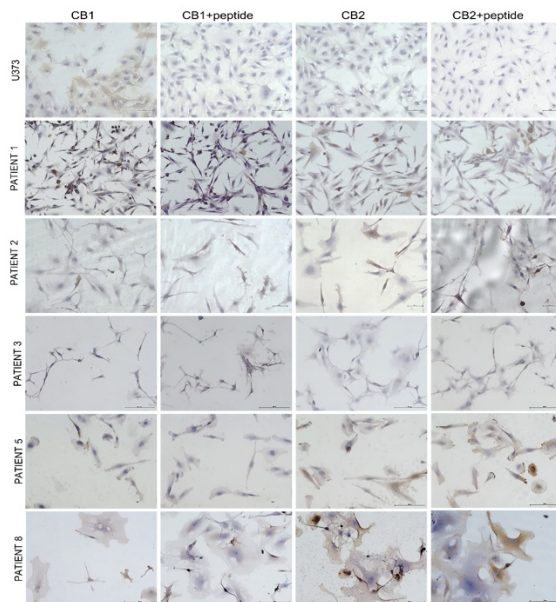


Figure 2: CB1 and CB2 are differentially expressed in GB cells. Immunocytochemical staining of CB1 and CB2 receptors (brown) in GB tissue samples. Cell nuclei were counter stained using haematoxylin (blue). Scale bar represents 50µm.

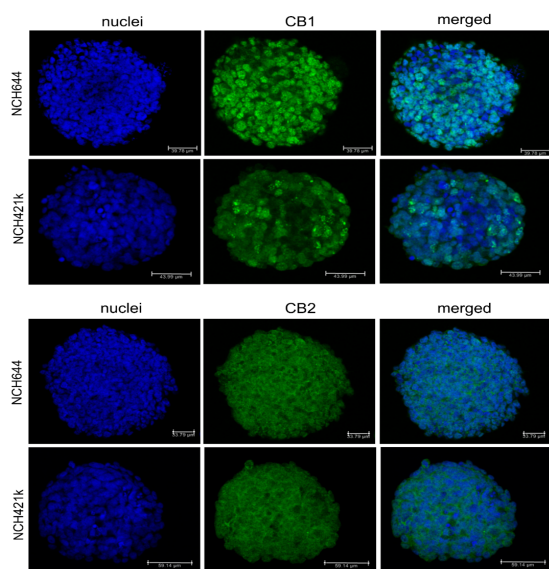


Figure 3: CB1 and CB2 are differentially expressed in Glioblastoma stem cells (GSC) B cells. Immunocytochemical staining of CB1 and CB2 receptors (green) in GSC cells. Cell nuclei were counter stained by DAPI (blue). Scale bar represents 50µm

b) Quality analyses of tested compounds

Contracting Entity provided, natural cannabidiol (**CBD^M**) and delta 9-tetrahydrocannabinol (**THC^M**) dissolved in DMSO (Dimethyl sulfoxide) in 5mM (received on 29.3.2019 and 4.6.2019) and 50 mM (received on 4.6.2019) concentrations.

The stability of the compounds was analysed in three different time points by **Contracting Entity** (Irena Pribošič, PhD) prior the experimental application with HPCL method and **defined as pure and stable** (scans of HPLC analyses are **Attached 2.3.4.**), as shown in Table 2.

Analysis #	Date	CBD (mg/l)	THC (mg/l)	other
40	29.03.2019	1484	1524	bdl
54	30.5.2019	1442	1552	bdl
55	04.06.2019	1340	1422	bdl

bdl= bellow detection limit

c) We have tested CBD^M and THC^M of different concentrations to follow viability in GB tumour cells U373 (Figure 2).

Experimental

Viability of GB cell line U373

was determined after 48 hours of treatment with CBD^M and THC^M using the MTT reagent [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium-bromide; Sigma-Aldrich, USA] according to manufacturer's instructions. Briefly, cells were seeded into 96-well plates (8000 cells/ well) and grown overnight. different concentrations of CBD^M and THC^M (0-50 µM). Stock solutions of CBD^M and THC^M were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Control incubations contained the same amount of DMSO (0.2 %, v/v) as in all the treatment conditions. After 48 h, MTT was added. After 3 h incubation, the formazan crystals were dissolved in DMSO and the absorbance was measured as the change in optical density (ΔOD 570/690 nm) using microplate reader (Synergy™ HT, Bio-Tec Instruments Inc., USA). Cell viability was analysed using GraphPad Prism software.

d) We have tested CBD^M and THC^M of different concentrations to follow viability in GSC tumour cells NCH644 and NCH 421k (Figure 4).

Experimental

Cell viability of glioblastoma stem cells NCH 644 and NCH 421k was determined after 48 hours of **treatment with CBD^M and THC^M** using the MTT reagent [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium-bromide; Sigma-Aldrich, USA] according to manufacturer's instructions. Briefly, cells were seeded into 96-well plates (10000 cells/ well) and grown overnight. Cell were treated as described above.

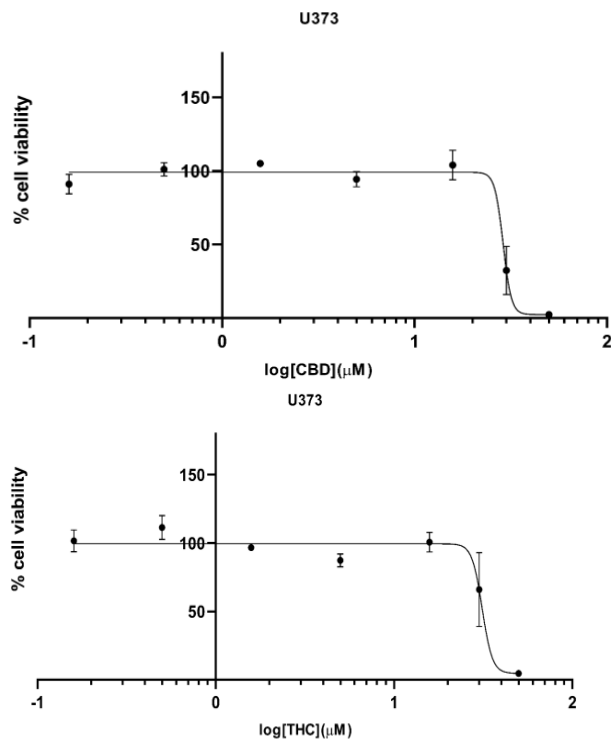


Figure 4: Cell viability of U373 glioblastoma cells after CBD^M and THC^M treatment. Each value represents the mean \pm SD ($n = 3$).

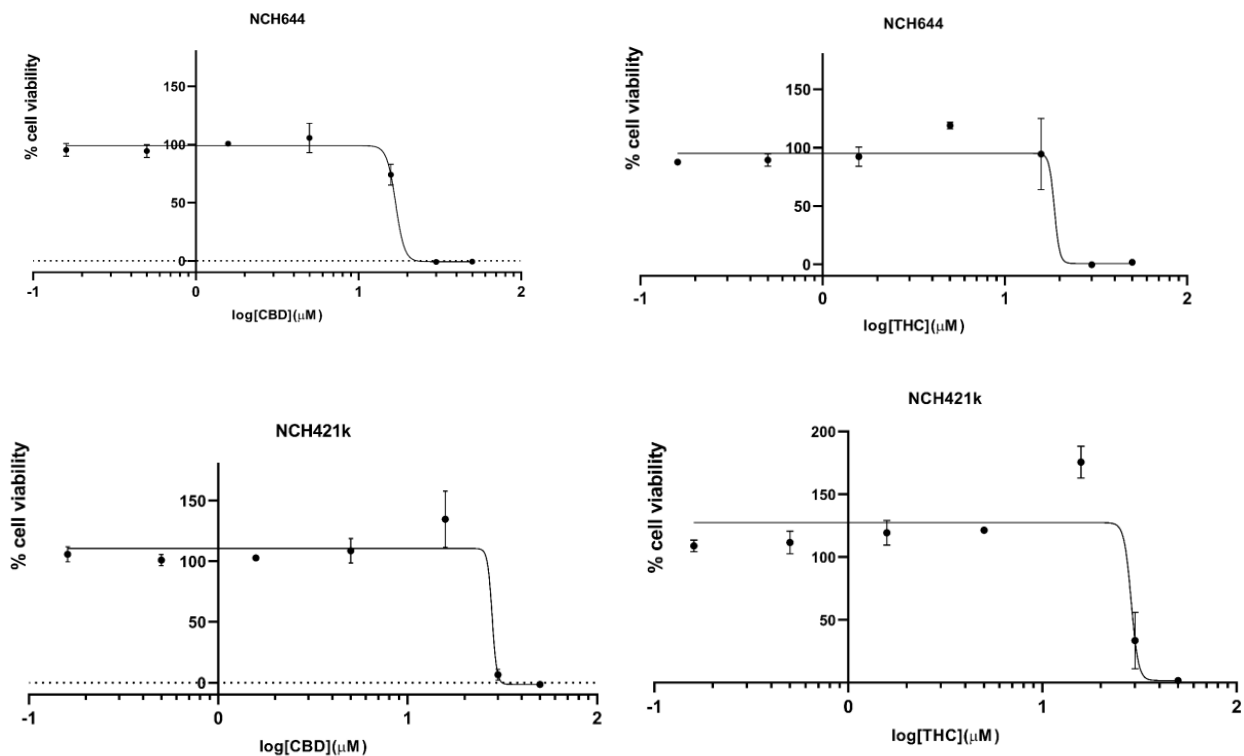


Figure 2: Cell viability of GSC cells NCH 644 and NCH 421k after CBD^M and THC^M treatment.
Cell viability was measured with MTT assay after 48h treatment. Each value represents mean \pm SD (n = 3).

Summary of IC50 Values

Table 2: Quantitative IC50 values for CBD^M and THC^M treatment of GB and GSC cells.

Cell line type	IC50 CBD	IC50 THC
NCH644 (GSC)	15.92 μ M	22.27 μ M
NCH421k (GSC)	27.93 μ M	28.67 μ M
U373 (GB)	29.72 μ M	34.94 μ M

IC50 values of the CBD^M and THC^M compounds were calculated as described by Deng, (2018) from the half maximal inhibitory effects on inhibiting cell viability of U373 and GSCs cell lines using GraphPad Prism software. We found that CBD^M reduced cell viability of NCH 421k and U373 cells with similar potencies with IC50 between 28 and 30 μ M. NCH 644 was more sensitive for CBD^M treatment with IC50 around 16 μ M. THC^M was less potent for cell viability, with IC50 values between 23 and 35 μ M. Further analyses on glioblastoma cells isolated from the patient tumour samples are being in progress.

The results obtained with MGC preparation are in the range of the published data by several other authors, such as Deng et al.(2017) , Nabissi et al., (2015) and Torres et al. (2011). At present we cannot conclude that the GSC cells are more susceptible for THC /CBD vs differentiated GB cells, where on the basis of previous data we suspected that this would be the case. Noteworthy the Cannabinoids effects in GSC was measured in 3 D cultures where the diffusion rate in the cells is lower than in 2 D cultures

References

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