THE RATIONALE, DESIGN AND METHODS OF NEW STUDIES

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Evaluation of efficacy and mechanisms of action of Cannabis sativa extracts with analgesic, anti-inflammatory and antiemetic properties in an *in vivo* model

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ABSTRACT

University of Medical Sciences participates in the realization of the project titled: „Development of the technology of producing cannabinoids from low THC hemp for use as preparations supporting treatment in oncological patients" awarded by the National Centre for Research and Development under project number: INNOMED/I/11/NCBR/2014. The duration of the grant is 36 months, and the total value of the grant is 28011845 PLN. The project is run by University of Life Sciences in Poznan.
Laboratory of Experimental Pharmacogenetics at the Department of Clinical Pharmacy and Biopharmacy, Poznan University of Medical Sciences (PUMS) is realizing the task number 4 titled "Evaluation of efficacy and mechanisms of action of Cannabis sativa extracts with analgesic, anti-inflammatory and antiemetic properties in an in vivo model." The aim of this project is the development of cannabinoid extract with reduced psychoactive component (THC), which due to its high content of cannabidiol (CBD) is meant to provide analgesic properties, and at the same time to reduce the risk of addiction and overdose. University of Medical Sciences is evaluating the analgesic, anti-inflammatory and antiemetic properties of the extract of *Cannabis sativa* in animal models coupled with neuropathic pain. Pharmacodynamic effects of plant extracts will be later assessed taking into account the level of selected genes and proteins expression..

Keywords: pharmacogenomics, gene expression level, neuropathic pain, cannabinoid.

General information

Research task No. 4 entitled "Evaluation of efficacy and mechanisms of action of Cannabis sativa extracts with analgesic, anti-inflammatory and antiemetic properties in an in vivo model" is realized within the framework of the research project entitled "Development of the technology of producing cannabinoids from low THC hemp for use as preparations supporting treatment in oncological patients" awarded by the National Centre for Research and Development under project number: INNOMED/I/11/NCBR/2014. The duration of the grant is 36 months, from 2014-09-01 to 2017-08-31 and the total value of the grant is 28,011,845 PLN, the total cost of the task No 4 is 880,000 PLN. The project is run by University of Life Sciences in Poznan with the head of the project, prof. Ryszard Słomski. The project titled: „Development of the technology of producing cannabinoids from low THC hemp for use as preparations supporting treatment in oncological patients" will be based on cooperation between partners that have established long term cooperation i.e. Poznan University of Life Sciences (PULS), Department of Biochem-

istry and Biotechnology, Institute of Natural Fibres and Medicinal Plants (INF&MP), Poznan University of Medical Sciences (Poznań Laboratory of Experimental Pharmacogenetics, Department of Clinical Pharmacy and Biopharmacy) (PUMS), Institute of Human Genetics PAS (IHG), Laboratory of Molecular Genetics (LMG), and PozLab Ltd.

Establishing of the consortium composed of the above mentioned organizations aims at timely completion of the project tasks and reaching the implementation stage for a standardized hemp extract prepared for pre-clinical trials according to the registration procedures for a medical product. The main project team realized task No 4 consists of: head of the task No 4 dr hab. Agnieszka Bienert and co-investigators: prof. Przemysław Mikołajczak, prof. Edmund Grześkowiak and dr Joanna Bartkowiak-Wieczorek. Local Ethics Committee of the Use of Laboratory Animals in Poznań permission number is nr 42/2015, 3/2017 and 66/2017.

Research Project Objectives

Cancer is a growing health and economical problem of the global population. In Poland there are about 200,000 people needing treatment for cancer pain, and every year comes around 65,000 patients more. 30–50% of patients in the early and 70–90% in the advanced stage of the disease suffer from cancer pain, 42% suffer from poor control of the aliment; 23% experiencing moderate or severe pain does not get any pain treatment, 31% of patients suffer from this pain for more than one year and 64% of patients experience side effects of analgesic drugs [1]. Neurophatic pain (NP) occurs in as many as 90% of patients during cancer. The International Association for the Study of Pain defines neurophatic pain as pain initiated or caused by a primary lesion or dysfunction in the nervous system, thus NP may be caused by any disease or injury to the nervous system [2]. The basis of treatment of cancer pain remains pharmacological treatment. Recent clinical trials have demonstrated the current role of cannabinoids in refractory chronic and cancer pain [3]. Cannabinoids may be a useful addition to current analgesic treatments [4]. The project is in full accordance with the INNOMED program as it focuses on innovative production of a preparation for medical use, including cancer patients. It also meets the needs of society in this respect.

The main objective of this project is preparation of the cannabinoid extract with decreased content of the psychoactive ingredient (THC), having at the same time high levels of cannabidiol (CBD). Such extract would have analgesic characteristics, but simultaneously the risk of addiction and overdosage would be significantly decreased. Products available so far on the market, including both the cannabis extracts and synthetic medicines, display high content of THC, since they are dedicated to mitigate symptoms other than neuropathic pain – therefore the presence of psychoactive compound is desired [5]. As a result of innovative approach suggested in this project, cannabinoid extract with low concentration of THC could be applied in the treatment of cancer patients.

A major contribution to science will be brought by the study of expression of genes involved in the metabolism of cannabinoids in in vivo model. The aim of the research task No 4 is to assess an analgesic and anti-inflammatory activity of 2 varieties of *Cannabis sativa* with a low content of THC in animal models of pain. After detailed phytochemical analysis, pharmacodynamic activities of plant extracts, the gene (mRNA) and protein levels of the CB1, CB2, and the NMDAR2B receptors in hippocampus and frontal cortex as well as the Nf-kB, TNFα, COX1, COX2 in the brain, and peripheral blood lymphocytes, liver of rats will be examined. On the basis of these complex studies both the mechanisms of action of the extracts as well as their eventual use as the analgesic or antiemetic products will be proposed.

Additionally, bioavailability of cannabidiols from the examined extracts will be assessed in an animal model.

Research plan

There are some evidence provided both from animal and clinical studies on the efficacy of analgesic effect of different cannabinoid compounds, both hallucinogenic (delta9-tetrahydrocannabinol – THC) and non-hallucinogenic (cannabidiol, delta9- tetrahydrocannabivarin, beta-caryophyllene) [6]. In the project, some aspects of the analgesic and anti-inflammatory effects of *Canabis sativa* extracts containing non-psychotropic plant-derived cannabinoids seem to be interesting in context of discovery and development of drug for the treatment of neuropathic pain. At first the acute toxicity after oral administration of the extracts (according to OECD directions) will be performed with starting dose of the extract 2 g / kg b.w. intragastrically (i.g.) in mice. Moreover a conventional 28-day repeat dose toxicity test will be also assessed in mice

Animals will receive extracts with different concentrations of cannabidiol after induction of neuropathic pain by vincristine and acute inflammation by carra-

geenan. Next, the tail flick test and von Fray test will be performed in order to assess both typical central-mediated analgesic activity and peripheral response. The biological material derived from the animals will be examined in molecular and biochemical tests. This step of study will be focused on both, the relative CB1, CB2, and the NMDAR2B receptors mRNA and protein level changes analyses in hippocampus and frontal cortex as well as the Nf-kB, TNFα, COX1, COX2 mRNA and protein level changes analyses in peripheral blood lymphocytes of rats. The obtained results will give the answer which of the active compounds dominates in extracts and whether their concentration is coupled with the expression of above mentioned genes and proteins levels with interrelationship to pharmacodynamic activities.

Basic Concept

Clinical studies largely affirm that neuropathic pain patients derive benefits from cannabinoids treatment. Cannabinoids exert their antinociceptive effects by complex mechanisms involving also effects on the central nervous system. This is consistent with the anatomical location of CB1 receptors in areas relevant to pain in the brain. There is recent evidence implicating CB2 receptors in the antihyperalgesic activity of cannabinoids in models of acute and chronic neuropathic pain, especially of inflammatory origin. Moreover, there is considerable evidence that activation of NMDAR contributes to the mechanism of pathological pain, especially NR2B-containing NMDA receptor is one of the best potential targets for neuropathic pain. Also, it has been proved that inflammation process plays a crucial role in pain induction by activation of selected factors such as TNF, Nf-kB, COX1, COX2. Taking into account the neuropatic and inflammatory background of the pain the evaluation of analgesic, anti-inflammatory and antiemetic properties of Cannabis sativa extract in in vivo model will be analyzed. In addition, CB1, CB2, NMDAR2B, Nf-kB, TNFα, COX1, COX2 genes expression and proteins levels in hippocampus, frontal cortex, lymphocytes and liver of rats will be studied. On the basis of these studies the mechanisms of action and eventual use of the extracts as an analgesic or anti-inflammatory product will be proposed.

Research Methodology

Male Wistar rats (200–220 g) will be housed five per cage at constant temperature (22 ± 2°C), with a 12:12 h light/dark cycle, and free access to food and water at all times for at least a week before starting of the study. The experiments will be carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental pain in conscious animals. Acute inflammation will be induced by i.pl. injection of 0.1 ml carrageenan (1% w/v in saline) into the right paw. Extracts' doses calculated in relation to their contents of cannabidiol (5, 7.5, 10, 20, and 40 mg/kg, p.o.) or an appropriate volume of vehicle, will be administered orally after the induction of acute inflammation: 2 h after carrageenan on the 1st day and then on the 2nd and 3rd days at the same time as the first injection. Control animals will receive an i.p. injection of saline (0.1 ml) and oral doses of drug vehicle.

Neuropathic pain: To induce neuropathic pain, the animals will be administered for 5 days with anintraperitoneally (i.p.) dose of vincristine of 0.1 mg/kg m.c, followed by two days with saline by the same route in the corresponding volume (Aley et al. 1996, Bhalla et al. 2015). This cycle will be repeated twice. After induction of neuropathic pain, it will be treated over 5 days with the substances (examined extracts or standard substances).

Carrageenan-induced edema: The paw volume will be measured with a plethysmometer (Ugo Basile, Varese, Italy). On the 1st day, the volume will be measured directly before the injection of carrageenan or saline and then after 3, 5, 6, and 7 h. On the next 2 days, paw volumes will be recorded just before drug or vehicle injection, and on the last day (the 4th after carrageenan) just before euthanasia. Data will be expressed as changes of edema (difference in volume between the right and left paws).

Tail flick test

The analgesic effect of the substances (extracts and standard drugs) will be assessed by the tail-flick test using the apparatus Ugo Basile Tail Flick Test Apparatus. In this method the light beam will be directed to the rat's tail 2 cm of its end. The animals will be immobilized at a special cage with a tail protruding outside the cage and placed on a plastic pad. The time since the beginning of light stimulus until the tail withdrawal from the place of exposition on the light will be measured (latency or reaction time in seconds). In all groups immediately prior to the measurement substance administration time t = 0 will be determined. Then measurements will be obtained at 1, 2, 3, and 6 hours after administration of the substance. The maximal time of a tale light exposure after drug administration was established as 60 seconds.

Von Fray test

The test is used to evaluate sensory sensitivity. The test assesses a time of pain response to peripheral mechanical stimuli. The animal will be placed in a cage made of a transparent plastic material, wherein the substrate is a wire mesh having a mesh size of 0.5 cm. To adapt to conditions in the unit the animals will be placed in it three minutes before the measurement. At this time, the measurement will be performed (3 to 6) with a gradual increase in the force of the metal filament having a diameter of 0.5 mm at the plantar rear right paw of rat. The increase in force filament will be from 0 to 50 grams per 10 seconds. Endpoint measurement will be a paw withdrawal by the animal. In each group, six measurements will be made for (all of which arithmetic mean) in each rat – immediately before administration of the substance (time t = 0) and 1, 2, 3, and 6 hours after administration.

Antiemetic activity (Wang et al. 2005, Tatsushima et al. 2011, Shi 2014)

It is known that kaolin intake is a good preclinical screen for drugs that are antiemetic, therefore its consumption will be used as an indicator of pro- and anti-emetic activity (by Wang et al. 2005, Shi 2014). The mesh container of 30 g kaolin pellets will be placed on the wire mesh floor of the cage, along with 70 g normal feed for 3 days before the experiment, and the animals will allow to adapt to the presence of both containers. To measure the kaolin consumption during a 24-h period, the remaining kaolin in the container and kaolin spilled in the cage will be collected, dried, and weighed at 10:00 h every day. The amount of normal feed and water intake will be measured in the same manner as for kaolin intake. Fresh kaolin, water and normal feed pellets will be placed in these containers every day.. The rats with kaolin intake <1.0 g/day on the last of 3 days adaptation will be used for substance tests. The animals will be injected i.p. with vehicle (saline) or cisplatin (3 mg/kg) 60 min before the placement of new pellets and kaolin after adaptation (day 0). Ondansetron (reference substance, 2 mg/kg, i.p.) will be administered 1 h before (on day 0, 1 and 2 – three times in total) before administration of cisplatin. Extract in doses calculated in relation to the contents of cannabidiol (2.5, 5, 7.5, 10, 20, and 40 mg/kg, p.o.) or an appropriate volume of vehicle, will be administered acutely 1 h before cisplatin injection. The studies will be carried out only using the extract of Cannabis obtained from one set and one part of the plant – the most effective in studies of analgesic activity.

Biochemical studies: Four days after carrageenan injection, animals will be decapitated, brain (hippocamp, frontal cortex), peripheral blood, liver tissues will be collected, rapidly frozen in liquid nitrogen and stored at -80°C.

Locomotor activity

Locomotor activity will be evaluated using a Ugo-Basile apparatus (Varese, Italy) by placing animals in the centre of the apparatus and recording their horizontal and vertical activity (Mikolajczak et al., 2002). The data obtained will be expressed as signals corresponding to spontaneous movements for 5 minutes after previous 20 minutes habituation to the activity meter cage. Locomotor activity will be measured: 60 minutes after a single dose of the extract. Control groups will be treated according to the appropriate treatment schedule.

Motor coordination

The effect of extracts on motor impairment will be quantified with chimney test. In this test rats will able to climb backward up to the plastic tube. Motor impairment will be indicated by the inability of animals to climb backwards up the tube within 60 sec (Borowicz et al. 2002).

Molecular investigations on CB1, CB2, NMDAR2B, COX1, COX2, TNF α, and Nf-kB mRNA and protein level changes in the isolated biological material RNA.

Total RNA isolation from the rats' hind paw, brain (hippocamp, frontal cortex) and blood lymphocytes will be carried out using TriPure Isolation Reagent (Roche) according to manufacturer's protocol. The 1–2 µg of total RNA from all samples will be used for the reverse transcribed into cDNA using SuperScript First-Strand Synthesis System (Roche) according to manufacturer's protocol. The CB1, CB2, and the NMDAR2B receptors genes in hippocampus and frontal cortex as well as the Nf-kB, TNFα, COX1, COX2 mRNA levels in the paw and peripheral blood lymphocytes of rats will be analyzed by quantitative real-time PCR (qPCR) reaction using a LightCycler TM Instrument (Roche, Germany) and a LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science, Germany) according to the instructions of the manufacturer. An GAPDH and/or PBGD gene will be used as a housekeeping gene (endogenous internal standard) for normalizationof qPCR reaction.

Changes of proteins levels (Nf-kB, TNFα, COX1, COX2, NMDAR2B, CB1 and CB2) in a rat samples will be determined with immunoenzymatic technique (ELISA method) and/or Western-Blot analysis using com-

mercial kits, buffers and antibodies. In the experiment homogenized and lysed (heart, aorta and blood lymphocytes) samples taken from animals immediately after decapitation will be used, followed by centrifugation at 2000 rpm for 10 minutes to obtain a supernatant. On the basis of these data a precise correlation between the observed quantitative changes in mRNA and proteins will be made. Knowing these relations can significantly the mechanism of action of studied plant extracts.

Measurable Effects

The proposed project is closely innovative. The very idea is innovative, because Cannabis extract with reduced THC content intended for biomedical purposes has not yet been developed, what provides applicants with a possibility to enter the market with a wide offer of a global scope. Results of pharmacological and pharmacogenetic studies will broaden the knowledge about content of nonpsychotropic cannabinoids in the Canabis sativa extracts and their analgesic and anti-inflammatory activities in animal models coupled with gene expression and protein level of CB1, CB2 and NMDAR2B receptors and Nf-kB, TNFα, COX1, COX2 in rats. On the basis of these complex studies the mechanisms of action will be proposed. The results would be a starting point for planning a future medicinal product used for alleviation of harmful pain (e.g. neuropathic pain).

Expected Results

The results obtained during the realization of the task No 4 will allow understand the molecular mechanism of the influence of Canabis sativa on neuropatic pain thorough examination of the animal behavior and different gene expression level of selected genes and proteins: CB1, CB2 receptors and NfkB, TNFα, COX1, COX2 in rats. Taking into account a number of scientific data showing the occurrence of cannabinoid receptors in high density in many areas related to pain [8; 9] and suggesting that cannabinoids inhibit cyclooxygenase enzymes COX1 and COX2 [10]. We expect that different doses of Canabis interact with examined receptors in brain, limphocytes, paw and liver in different manner.

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Conflict of interest statement
The authors declare no conflict of interest.

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