Analysis of the effects of BZO-HEXOXIZID (MDA19) on the expression level of PLA2G7, UCP2 and NEDD4L proteins in prostate cancer cells

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Received November 19, 2024; Accepted February 12, 2025

DOI: 10.3892/wasj.2025.333

Abstract. Prostate cancer (PCa) is a hormone-dependent type of cancer that exhibits oncogenic modulated metabolic programming, utilizing lipid oxidation for energy. Previous studies have demonstrated that the increase in lipid droplets (LDs) during cancer progression is related to increased cancer aggressiveness and chemotherapeutic resistance. Cannabinoids have been shown to exert anti-tumorigenic activities, such as inhibiting tumor cell proliferation, accelerating apoptosis, and inhibiting cell migration and angiogenesis. The aim of the present study was to determine the effects of MDA19 on phospholipase A2 group VII (PLA2G7), uncoupling protein 2 (UCP2) and NEDD4 like E3 ubiquitin protein ligase (NEDD4L) proteins involved in the formation of LDs in DU145 and PC3 PCa cell lines. The IC_{50} value of MDA19 determined using MTT assay was used to detect the protein expression levels of PLA2G7, UCP2 and NEDD4L by ELISA. It was observed that the protein levels of PLA2G7 and UCP2 decreased significantly ($P<0.0001$). However, the NEDD4L protein level increased significantly in a time-dependent manner in the DU145 and PC3 PCa cell lines. The results of the present study indicate that MDA19 exerts an inhibitory effect on the protein expression levels of PLA2G7 and UCP2, which are involved in the formation LDs, and also exerts a promoting effect on the protein expression level of NEDD4L. Thus, MDA19, a bioactive cannabinoid analogue, may prove to useful as an alternative approach for the treatment of PCa. Further studies targeting cancer metabolism with cannabinoids and their derivatives are required to provide novel strategies for the prevention and treatment of cancer.

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Key words: cannabinoids, lipid droplets, MDA19, phospholipase A2 group VII, NEDD4 like E3 ubiquitin protein ligase, prostate cancer, uncoupling protein 2

Introduction

Cancer is a disease in which some of the cells in the body grow uncontrollably; it is influenced by genetic and environmental conditions (1). Prostate cancer (PCa) is considered a hypoxic and lipogenic tumor (2) and it is the second most prevalent type of cancer among males and also one of the leading causes of cancer-related mortality worldwide (3,4). Lipids are involved in signal transmission, maintaining the structural integrity of cellular membranes, and regulating energy metabolism (5). The role of lipid and glucose metabolism in PCa differs from other types of cancer. While the majority of cancers primarily use glycolysis, PCa uses lipid metabolism as opposed to glycolysis for energy metabolism. Cancer cells may increase lipid uptake and lipid synthesis due to increased energy demands (6). The increased amount of lipids leads to the accumulation of lipid droplets (LDs) in the cells. A LD is a type of functional organelle in the cell, consisting of a neutral lipid nucleus, a single-layer phospholipid membrane and LD-associated proteins. LDs are involved in diverse biological phenomena, including cell proliferation, apoptosis, lipid metabolism, stress, immunity and signal transduction (7). LDs are known to be involved in fundamental processes of cell homeostasis and have been shown to be associated with various disorders, including metabolic diseases, inflammatory reactions in leukocytes and cancer development (8). Studies have shown that an increase in LDs is associated with cancer cell proliferation and chemotherapy resistance (7). Other studies investigating the role of lipids in cancer progression have revealed that aggressive cancers can meet their lipid requirements independently from circulation by synthesizing high rates of *de novo* fatty acids (FAs) (8,9). At the same time, cancer cells enter into a symbiotic association with tumor-associated adipocytes, where the lipolysis of LD in adipocytes provides FA to cancer cells for energy production (7-9). In PCa, there are some proteins that play a role in lipid metabolism and the

formation of LDs, such as the phospholipase A2 (PLA2) group VII (PLA2G7), uncoupling protein (UCP)2 and NEDD4 like E3 ubiquitin protein ligase (NEDD4L) proteins. PLA2G7 is a 45-kDa monomeric protein and is a PLA2 involved in phospholipid catabolism (10,11). Phospholipases are a family of lipid-modifying enzymes that break down phospholipids and regulate bioactive lipid levels (12). PLA2s function as: i) Suppliers of free FAs from membrane phospholipids for the synthesis of neutral lipids; ii) producers of metabolites that can control LD metabolism; and iii) direct modulators of organelle formation (8). Additionally, it has been shown that the anti-proliferative effect of PLA2G7 gene silencing is enhanced by lipid-reducing statins in PCa cells (13). UCPs belong to the family of mitochondrial inner membrane transporters (14). These proteins are involved in the function of the mitochondrial membrane and cellular energy regulation. The UCP group has five members: UCP1, UCP2, UCP3, UCP4 and UCP5, which are distributed in different tissues in the body (15). UCP2 plays a crucial role in cancer metabolism and supports tumor growth and survival (16). Additionally, it has been suggested that the switch from glucose oxidation to FA oxidation may be supported by UCP2 (17). NEDD4L is an E3 ubiquitin ligase described to be involved in a variety of cellular activities by regulating substrate ubiquitination (Ub) and protein degradation (18). NEDD4L is involved in the regulation of tumor cell functions, such as proliferation, apoptosis, migration, invasion, epithelial-mesenchymal transition and drug resistance by controlling protein degradation through Ub (19).

Hemp (*Cannabis sativa* L.) is a plant that has been used in the treatment of various diseases since ancient times. Cannabinoids are active compounds produced by *Cannabis sativa* L. and its species. These are classified in three main groups: Phytocannabinoids, endocannabinoids and synthetic cannabinoids (20). The function of cannabinoids is mediated through the endocannabinoid system, including cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). Some synthetic CB1 and CB2 agonists have been demonstrated to inhibit tumor progression and metastasis (21). The substance known as the most potent and main component in hemp is Δ9-tetrahydrocannabinol (THC) (20). Benzohydrazide N'-[(3Z)-1-(1-heksil)-2-okso-1,2-dihidro 3H-indol 3-iliden] benzohidrazid (MDA19), an analogue of THC, is known to be a selective agonist at CB2 (4,21).

To date, to the best of our knowledge, there is no study available in the literature examining the effects of MDA19 on PLA2G7, UCP2 and NEDD4L proteins, which play a role in LD metabolism in PCa. The present study thus aimed to determine the therapeutic efficacy of MDA19 in the treatment of PCa, and to contribute to the literature by demonstrating its effects on LD metabolism, an alternative pathway of cancer metabolism.

Materials and methods

Cell lines and cell culture. The human metastatic prostate carcinoma cell lines, DU145 (ATCC HTB-81) and PC3 (EACC 95012614), were obtained from the Department of Biotechnology, Ankara University (Ankara, Turkey). The cells were cultured at 37˚C and 5% CO_2 using high-glucose DMEM (SLD-524-500, Serox GmbH) containing 10% FBS (FBS-11B, Capricorn Scientific), 1% penicillin-streptomycin (15140122; Gibco; Thermo Fisher Scientific, Inc.), and 1% non-essential amino acid (L-glutamine) (SRL-810-100; Serox GmbH).

Cell lysate preparation. The cell lysate was prepared by applying the freeze-thaw method. In the first step, the medium in the 96-well well plate was removed and washed with PBS (PBS-1A, Capricorn Scientific). Cells treated with trypsin (Sartorius 223601012) were neutralized by adding a medium (DMEM, Serox GmbH). The cells were centrifuged at 1,400 x g for 3 min at 4˚C. The supernatant was removed and washed three times with cold PBS. Following each wash, the supernatant was discarded by centrifugation at 3,900 x g at 4˚C for 2 min. Subsequently, 200 μl PBS were added and maintained at -80˚C for 5 min and incubated at 37˚C for 5 min. This procedure was replicated three times. Cell lysates were stored at -20˚C.

Preparation of BZO-HEKZOKZID (MDA19). The molecular weight of MDA19 (HY-15451, MedChemExpress), whose molecular formula is $C_{21}H_{23}N_3O_2$, is 349.43 g/mol. The stock concentration of MDA19 was calculated to be 10 mM and was prepared for use by dissolving it in dimethyl sulfoxide (DMSO) (1.167.431.000; MilliporeSigma).

Evaluation of cell viability and proliferation. MTT assay, one of the colorimetric methods for the detection of cytotoxicity, was used to determine the effects of MDA19 on the viability and proliferation of DU145 and PC3 cells. To determine the concentration at which MDA19 killed 50% of the cells, various concentrations (0, 25, 50, 100, 150 and 200 μM) of MDA19 were applied to the cells cultured in 96 well-plates for 24, 48 and 72 h. Subsequently, 5 mg formazan, the reagent of MTT, was dissolved in 1 ml PBS. Subsequently, 10 μl MTT reactive (20395.02; Serva Electropheresis GmbH) was applied to each well and after 2 h, the solvent was added for overnight incubation at 37˚C, and the absorbance was measured at a wavelength of 570-690 nm (Epoch 2, BioTek Instruments, Inc.). The reagent of MTT, formazan, was dissolved in PBS. IC_{50} values of MDA19 were calculated using the GraphPad Prism 8.4.2 program (Dotmatics).

Determination of the expression levels of PLA2G7, UCP2, and NEDD4L proteins using enzyme-linked immunosorbent assay (ELISA). Measurements were made using ELISA to determine the protein expression levels of PLA2G7, UCP2 and NEDD4L. For ELISA, the PLA2G7 ELISA kit (E3993Hu, Bioassay Technology Laboratory), UCP2 ELISA kit (E5683Hu, Bioassay Technology Laboratory) and NEDD4L ELISA kit (E7630Hu, Bioassay Technology Laboratory) were used. While setting up the ELISA, the standards were prepared by diluting them at a ratio of 1:2. Subsequently, 50 μl standard solution, 40 μl cell lysate, 10 μl protein-specific biotinylated antibody, and 50 μl streptavidin-HRP were added to the wells, respectively and incubated for 60 min at 37˚C. Following incubation, washing was performed five times with washing solution; 50 μl substrate solution A and 50 μl substrate solution B were then added followed by incubation for 10 min at 37˚C. Finally, 50 μl stop

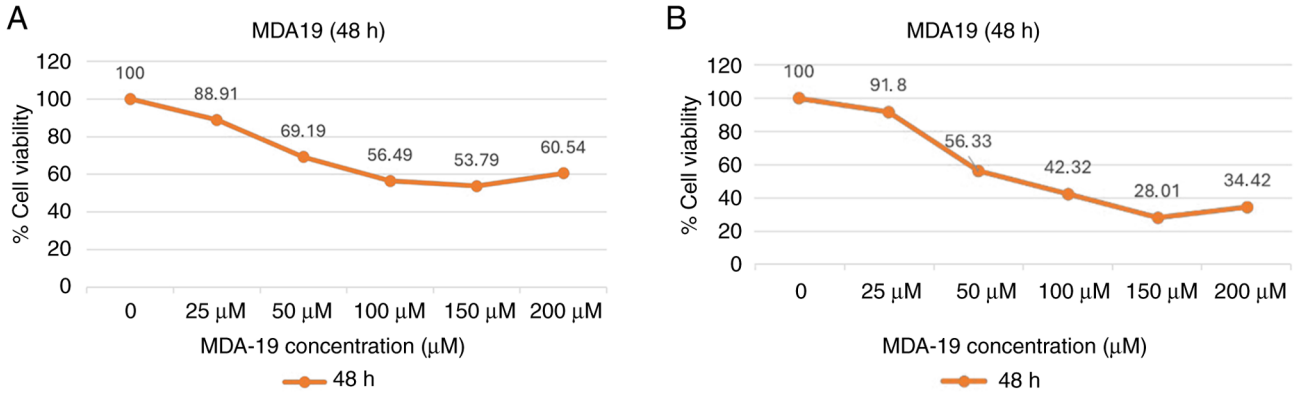

Figure 1. (A) Graphs illustrating the percentage viability of DU145 PCa cells treated with MDA19 for 48 h. (B) Graphs illustrating the percentage viability of PC3 PCa cells treated with MDA19 for 48 h.

solution was applied and the absorbance was measured at 450 nm (Epoch 2, BioTek Instruments, Inc.).

Statistical analysis. One-way ANOVA was used for the comparison of the ELISA results. When any differences were found, post-hoc pairwise comparisons were performed with the Dunnett's test to compare the control group with the experimental groups. Adjusted P-values were used for controlling the type I error rate in statistical decisions. Statistical analysis was performed for using GraphPad Prism 8.4.2 software (Dotmatics). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Anti-proliferative effects of MDA19 treatment. MDA19 was applied to the DU145 and PC3 PCa cell lines at the concentrations of 0, 25, 50, 100, 150 and 200 μM for 24, 48 and 72 h. The results of MTT assay revealed that the IC_{50} value at 48 h of MDA19 treatment was statistically significant. Thus, only the images for 48 h of MDA19 treatment are presented in Fig. 1.

Reduction of the protein expression level of PLA2G7. MDA19 was applied to the DU145 and PC3 PCa cell lines at the concentration of 90 μM for 24, 48 and 72 h and ELISA was then performed to determine the protein expression level of PLA2G7 in the DU145 and PC3 PCa cell lines (Fig. 2). Significant differences were observed in the protein expression level of PLA2G7 in the DU145 cell line. In the DU145 cells, in the absence of MDA19 treatment (MDA19-), the protein expression level of PLA2G7 was determined at 2,680 ng/l, and at 24 h of treatment with MDA19 (MDA19+ 24 h), it was determined at 2,599.5 ng/l. There was no significant differences detected in the protein expression level of PLA2G7 between these groups (P=0.0569). The protein expression level of PLA2G7 at 48 h treatment of MDA19 (MDA19+ 48 h) was measured at 2,124 ng/l. It was determined that there was a decrease in the protein expression level PLA2G7 compared to the DU145 (MDA19-) group (P<0.0001). The protein expression level of PLA2G7 was determined at 2,490 ng/l at 72 h of treatment with MDA19 (MDA19+ 72 h). A decrease in the protein expression level of PLA2G7 was detected compared to the MDA19- group (P=0.0028) (Fig. 2A).

In the PC3 cell line, in the MDA19- group, the protein expression level of PLA2G7 was determined at 2,686.5 ng/l, and at 24 h of treatment with MDA19 (MDA19+ 24 h) it was determined at 2,595 ng/l. A considerable difference was detected between these groups (P=0.0089). The protein expression level of PLA2G7 at 48 h of treatment with MDA19 (MDA19+ 48 h) was determined at 1,584 ng/l and a significant decrease in the protein expression level of PLA2G7 was detected compared to the MDA19- group (P<0.0001). At 72 h of treatment with MDA19 (MDA19+ 72 h) the protein expression level of PLA2G7 was determined at 2,901.06 ng/l. An increase in the protein expression level of PLA2G7 was detected compared to the MDA19- group (P=0.0003) (Fig. 2B).

Reduction of the protein expression level of UCP2. MDA19 was applied to the DU145 and PC3 PCa cell lines at the concentration of 90 μM for 24, 48 and 72 h and the results of ELISA revealed that the protein expression level of UCP2 exhibited significant differences in the DU145 and PC3 cell lines (Fig. 3). As shown in Fig. 3A, in the DU145 cells, the protein expression level of UCP2 was found to differ between the following groups: MDA19- and MDA19+ at 24, 48 and 72 h of treatment with MDA19. The protein level was determined at 2,486.13, 2,339.5, 1,496 and 2,094.88 ng/l at 0, 24, 48 and 72 h, respectively. According to the results of the analysis, a significant difference was detected in the protein expression level of UCP2 between the MDA19- group and at 24 h of treatment with MDA19 (MDA19+ 24 h) (P=0.0184). When the MDA19- group and the 48-h treatment group (MDA19+ 48 h) were compared, a significant decrease was detected in the protein expression level of UCP2 (P<0.0001). It was also determined that there was a decrease in the protein expression level of UCP2 between the MDA19- group and the 72-h treatment group MDA19 (MDA19+ 72 h) (P=0.0005; Fig. 3A).

In the PC3 cell line, the protein expression level of UCP2 exhibited similar changes following treatment with MDA19 at 24 and 48 h, and the protein expression level of UCP2 was increased at 72-h time point. The protein expression level of UCP2 was determined at 2,494.5 ng/l for the MDA19- group and 1,791.17 ng/l for the 24-h treatment group (MDA19+ 24 h), and the difference was significant (P=0.0037). For the 48-h treatment group (MDA19+ 48 h), the protein expression level of UCP2 was measured at 1,299.5 ng/l, and a

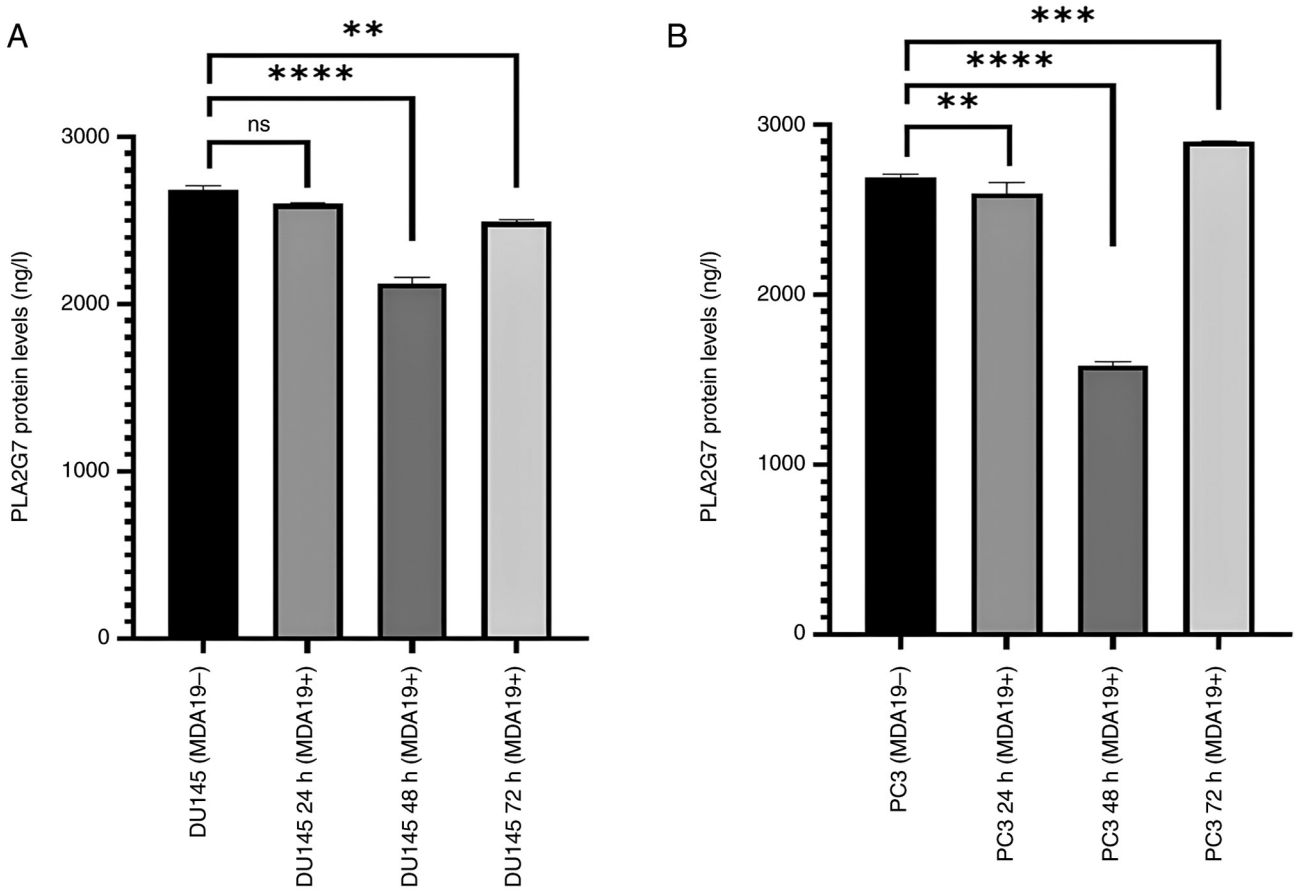

Figure 2. (A) Representation of PLA2G7 protein level in DU145 cells. ns, not significant (P=0.0569); **P=0.0028 and ****P<0,0001. (B) Representation of PLA2G7 protein level in PC3 cells. **P=0.0089, ***P=0.0003 and ****P<0.0001. PLA2G7, phospholipase A2 group VII.

significant difference in the protein expression level of UCP2 was observed between the MDA19- group and the MDA19+ 48 h group (P=0.0002). In the 72-h treatment group (MDA19+ 72 h), the protein expression level of UCP2 was determined at 2,025.5 ng/l and no significant difference was detected in the protein expression level of UCP2 compared to the MDA19- group (P=0.1844) (Fig. 3B).

Increase in the protein expression level of NEDD4L. MDA19 was applied to the DU145 and PC3 PCa cell lines at the concentration of 90 μM for 24, 48 and 72 h and the results of ELISA revealed that the protein expression level of NEDD4L exhibited significant differences in the DU145 and PC3 cell lines (Fig. 4). In the DU145 cell line, the protein expression level of NEDD4L was measured in the absence of MDA19 treatment and at the 24, 48 and 72-h treatment time points. NEDD4L expression was determined at 282.23, 302.98, 330.73 and 248.61 ng/ml at 0, 24, 48 and 72 h, respectively. A significant difference was detected in terms of the protein expression level of NEDD4L between the MDA19- group and the 24-h treatment group (MDA19+ 24 h) (P=0.0009). There was a significant increase in the protein expression level of NEDD4L between the MDA19- group and the 48-h treatment group (MDA19+ 48 h) (P<0.0001). It was also determined that there was a decrease in the protein expression level of NEDD4L between the MDA19- group and the 72-h treatment group (MDA19+ 72 h) (P=0.0002; Fig. 4A).

The protein expression level of NEDD4L in the PC3 cell line is presented in Fig. 4B. For the MDA19- group and the 24-h treatment group (MDA19+ 24 h), the protein expression level of NEDD4L were determined at 148.11 and 154.73 ng/ml, respectively, and a significant difference was detected (P=0.0031). For the 48-h treatment group (MDA19+ 48 h), the protein expression level of NEDD4L was determined at 183.86 ng/ml, and an increase in the protein expression level of NEDD4L was detected compared with the MDA19- group (P<0.0001). For the 72-h treatment group (MDA19+ 72 h), the protein expression level of NEDD4L was determined at 205.98 ng/ml, and an increase in was detected compared with the MDA19- group (P<0.0001) (Fig. 4B).

Discussion

The present study examined the effects of the cannabinoid analogue, MDA19, on the protein expression levels of PLA2G7, UCP2 and NEDD4L, which play a role in LD metabolism in metastatic PCa cell lines (DU145 and PC3) using ELISA. The results revealed that while treatment with MDA19 decreased the protein expression levels of PLA2G7 and UCP2, it increased those of NEDD4L.

PCa is considered a hypoxic and lipogenic tumor (2) and PCa cells change their metabolism to support their survival and metastasis (22). Apart from the classical metabolic changes highlighted by the Warburg effect, it has been shown that lipid

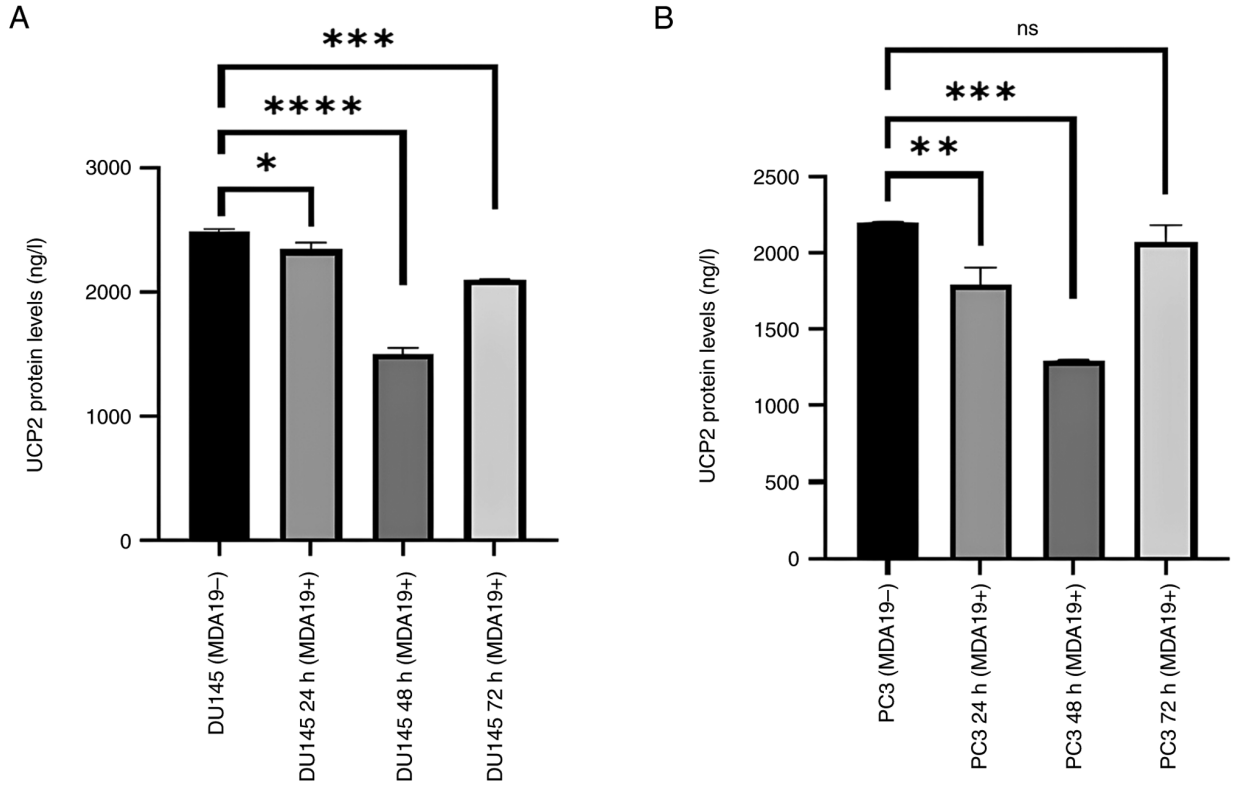

Figure 3. (A) Illustration of UCP2 protein level in DU145 cells. *P=0.0184, ***P=0.0005 and ****P<0,0001. (B) Illustration of UCP2 protein level in PC3 cells. **P=0.0037 ***P=0.0002; ns, not significant (P=0.1844). UCP2, uncoupling protein 2.

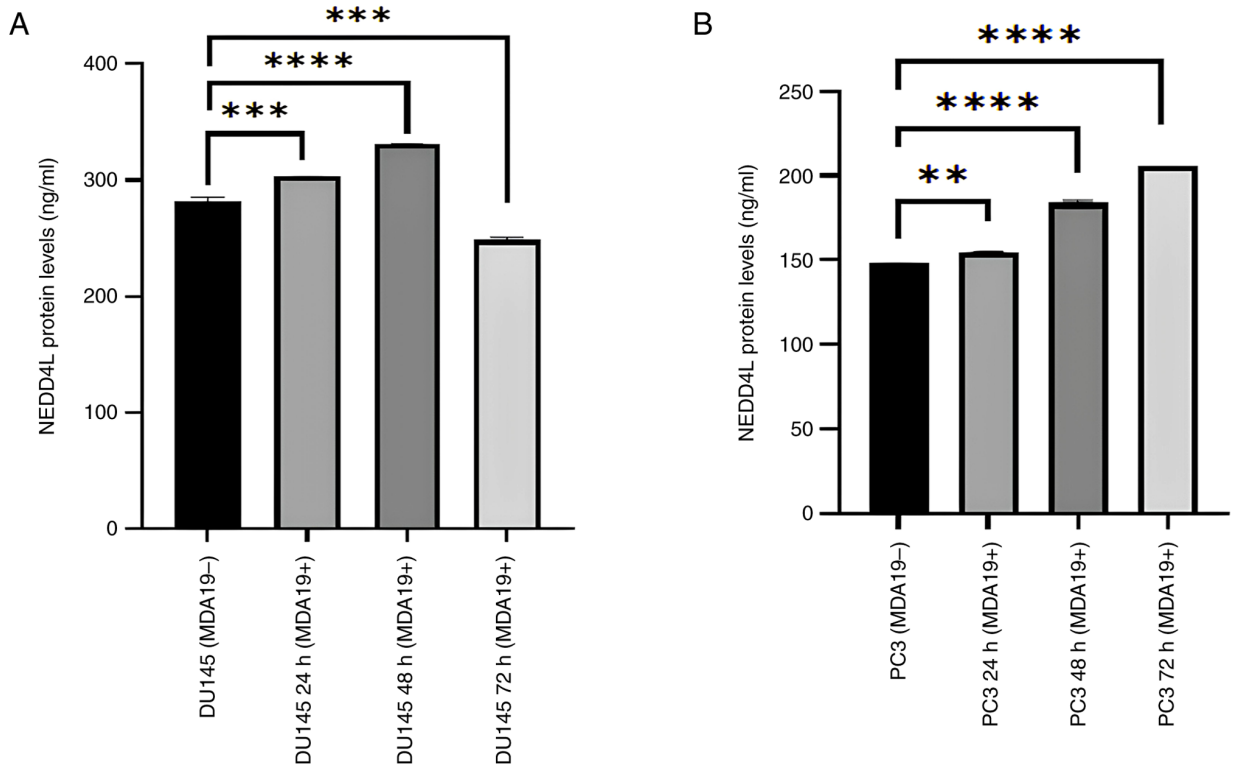

Figure 4. (A) Representation of NEDD4L protein level in DU145 cells. 24 h: ***P=0.0009 (24 h), ***P=0.0002 (72 h) and ****P<0.0001. (B) Representation of NEDD4L protein level in PC3 cells. **P=0.0031 and ****P<0.0001. NEDD4L, NEDD4 like E3 ubiquitin protein ligase.

metabolism is critical for tumorigenesis (23). These metabolic changes include various mechanisms, including LD storage, enhanced lipid uptake, *de novo* lipid synthesis and adjustments in lipolysis (24). Lipid deposition in LDs is a defining feature

of PCa cells (25). The synthesis and utilization of lipids in PCa cells are controlled by androgens. Androgen receptor signaling in PCa has been reported to upregulate the levels of lipid biosynthetic enzymes, such as fatty acid synthase (FASN) and acetyl-CoA carboxylase alpha (4). Additionally, Roman *et al* (25) demonstrated that lipid accumulation occurred in PC3 PCa cells in their study using the Raman mapping technique. The role of LD metabolism in the development of novel therapies of PCa has been recently studied (26). Thus PLA2G7, UCP2 and NEDD4L proteins play a critical role in LD metabolism in PCa.

Cannabinoids obtained from *Cannabis sativa L.* and its derivatives, used in the treatment of various diseases for palliative purposes in patients with cancer (27). Cannabinoids have been demonstrated to modulate metabolic reprogramming and inhibit cancer cell progress (28). Recent studies have shown that these compounds have effects on promoting apoptosis, arrest of the cell cycle, inhibition of cell migration and angiogenesis (27). In addition, cannabinoids affect AKT, EGFR and mTOR signaling pathways and play a role in cell growth, differentiation and metabolism (29). It has been stated that the stimulation of the PI3K/Akt signaling pathway regulates cholesterol uptake, glucose metabolism and lipogenesis through sterol regulatory element binding protein (SREBP), which supplies energy for rapid tumor growth. SREBPs play key roles in regulating cholesterol and lipid metabolism. The study by Sun *et al* (28) demonstrated that cannabinoids inhibit cancer cell progression by inhibiting the PI3K/Akt/mTOR pathway (27).

Due to the significance of lipid metabolism in PCa, studies on LD have become a popular in cancer treatment. As previously reported, PLA2G7 is a novel biomarker in 50% of primary PCa and 70% of metastatic PCa and is associated with the aggressiveness of cancer cells (30). PLA2s have appeared as key modulators of LD homeostasis and have been shown to regulate their formation at different levels (8). In their study, Atakol *et al* (31) observed that when the protein levels of PLA2G7 were compared in the DU145 and PC3 cells, the PLA2G7 protein level was higher in the DU145 cell line. However, no significant differences were detected (31). Jayaraman *et al* (32) provided a promoting force for LD formation as hydrolytic products were produced by PLA2G7, particularly free FAs (32). In the present study, when the time-dependent effect of MDA19 was examined in the DU145 and PC3 cell lines, it was demonstrated that the 90 μM concentration of MDA19 inhibited the two cell lines in a time-dependent manner. The highest inhibitory effect was observed at 48 h of treatment with MDA19 for the level of PLA2G7 in both cell lines compared to the MDA19- group. No significant change was observed in the level of PLA2G7 protein in the DU145 cell line at the 24-h time point; therefore, further studies on the effects of MDA19 on the PLA2G7 protein level are required.

The expression of UCP2, a regulator of cellular metabolism, has been found to be enhanced in a number of types of cancer, including leukemia, skin cancer, pancreatic cancer, colon cancer and hepatocellular carcinoma. A recent study demonstrated that targeting UCP2 inhibition in cancer treatments induces the apoptosis of tumor cells (33). UCP2 is involved in LD formation through FASN (7). In the study carried by Ke *et al* (34), UCP2 was shown to act as a critical regulator of lipid accumulation *in vivo* and *in vitro*, and the accumulation and synthesis of LDs were suppressed in UCP2-inactivated mice. Burch *et al* (35) investigated the UCP2 expression level using RT-PCR and western Blot analysis in non-malignant RC77N/E and malignant RC77T/E cells from prostate adenocarcinoma cells. As a conclusion of their study, it was found that the UCP2 protein level was significantly increased in malignant cells compared to non-malignant cells (36). Atakol *et al* (31) examined the protein level of UCP2 in the DU145 and PC3 cell lines. It was determined that the protein level of UCP2 in PC3 cells was higher than in DU145 cells (31). In the present study, when the effect of MDA19 on DU145 and PC3 cell lines was examined, it was observed that MDA19 had a significant time-dependent inhibitory effect on the UCP2 protein level. Compared with the MDA19- group, the highest inhibition of MDA19 on UCP2 expression was observed at 48 h in both cell lines.

Various E3 ubiquitin ligases, including NEDD4L, have been described as regulators of lipid machinery (36). The protein expression level of NEDD4L is lower in PCa samples compared to benign prostatic hyperplasia (18). In addition, Alberts and Rotin (37). noted that spartin activated NEDD4 family ligases for the degradation of LD proteins. Atakol *et al* (31) compared the protein expression level of NEDD4L in the DU145 and PC3 cell lines. The protein level of NEDD4L was found to be higher in DU145 than in PC3 cells in a time-dependent manner (31). In the present study, when the effect of MDA19 on DU145 and PC3 cell lines was examined, a significant time-dependent promoting effect of MDA19 on the protein level of NEDD4L was observed. The highest promoting effect was observed at 48 h of treatment with MDA19 in the DU145 cells (compared to the MDA19- group), while the optimal effect was observed at 48 and 72 h of treatment with MDA19 in the PC3 cells. It was hypothesized that MDA19 may regulate cancer cell proliferation by increasing the NEDD4L level in PCa. In the present preliminary study, the potential effects of MDA19 on the protein expression levels PLA2G7, UCP2 and NEDD4L, proteins involved in LD metabolism in metastatic prostate cell lines), were demonstrated.

To date, to the best of our knowledge, there is no study available in the literature demonstrating the effects of the cannabinoid analogue, MDA19, on LD metabolism in PCa. Thus, the present study examined the effects of MDA19 on the protein expression levels of PLA2G7, UCP2 and NEDD4L implicated in LD metabolism. In conclusion, it was determined that MDA19 exerts an inhibitory effect on the protein expression levels of PLA2G7 and UCP2, and a promoting effect on the protein expression level of NEDD4L. The findings of the present study suggest that targeting proteins, such as PLA2G7, UCP2 and NEDD4L, involved in LD metabolism, by treatment with MDA19 in PCa may reduce the proliferation of tumor cells and may provide novel treatment options in cancer. MDA19 may prove to be a novel alternative treatment option for PCa.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Ankara Yildirim Beyazit University Project Coordination Application and Research Center (grant no. 2432).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ES, SS, OOG and MEE were involved in the conceptualization of the study. ES, SS, AS, DA, HAK and EDK were involved in the study methodology (cytotoxicity test, MTT assay and ELISA). ES and SS were involved in literature review. ES, SS, OOG and MEE were involved in the formal analysis. ES and SS were involved in the writing and preparation of the original draft of the manuscript. ES, SS, OOG, AS, MEE, DA and HAK were involved in the writing, reviewing and editing of the manuscript. ES was involved in funding acquisition. ES and SS confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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