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Investigation the effects of synthetic cannabinoid-AKB48 on DNA methylation via the regulation of cannabinoid receptor gene specific methylation *in vitro*

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ABSTRACT: Synthetic cannabinoids have become a significant public health problem in the world because of inexpensive, easy to obtain, difficult to detect by many screening methods, constantly new compounds are produced and marketed, and so on. Despite their effect on Δ9-tetrahydrocannabinol (THC) like properties on cannabinoid receptors, they exert a very strong effect as opposed to THC and could cause serious side effects to death. Studies in the literature on causative epigenetic mechanisms of long-term usage of drugs like cocaine, cannabis, and heroin are available however, few studies have been conducted on synthetic cannabinoids, which are very common in use. Also, recently the endocrine disrupting effects of such substances have come into prominence. We aimed to examine potential effects of AKB48, one of the important synthetic cannabinoids, on cytotoxicity, global methylation and CpG island promoter methylation of cannabinoid receptor genes (*CB1* and *CB2*) and gene expressions of DNA methyltransferase enzymes (*DNMT1* and *MGMT*), *CB1* and *CB2* genes in prostate adenocarcinoma (PC-3) cells following 24 h exposure. In MTT assay, IC_{50} value was calculated as 343.8 µM for AKB48 in PC-3 cells. Percentage of 5-methylcytosine (5-mC%) levels were significantly decreased by 27% at 100 µM concentration. No significant alteration was shown in the promoter methylation of *CB1* gene compared to the control. These results will contribute to literature for determination of potential effects of synthetic cannabinoids on epigenetic modifications in the tissues of endocrine system.

KEYWORDS: AKB48; Cytotoxicity; DNA methylation; PC-3 cells.

1. INTRODUCTION

The cannabinoids act on the cannabinoid receptors (CB1 and CB2, specialized for endocannabinoids such as arachidonic acid, anandamide) in the organism. Cannabinoids have been divided into three classes: natural cannabinoids, endogenous cannabinoids and synthetic cannabinoids [1]. Marijuana, the plant-derived (natural) cannabinoid, is one of the psychotropic substances due to its narcotic properties which obtained from *Cannabis sativa subsp. indica* or *Cannabis sativa subsp. sativa* [2]. Because of a serial of pharmacological effects, it has been also used in the traditional medicine [2, 3].

Δ9-tetrahydrocannabinol (THC) is the best example of natural cannabinoids and the main component of hash. Synthetic cannabinoids are molecules synthesized in the laboratory to produce THC-like effects and have become popular among the world [4]. Abuse of commercial preparations of synthetic cannabinoids has been prominent for public health. Metabolites of synthetic cannabinoids could usually have higher receptor affinity compared to THC, so could result in more severe toxicological effects from induced by THC such as increased in morbidity and mortality originated from these active metabolites [5]. There are serious toxic effects of synthetic cannabinoids have been shown such as arrhythmias, myocardial infarction, sudden cardiac death, psychosis, suicidal ideation, epilepsy, acute tubular necrosis, and intracranial hemorrhage due to affecting various systems [6]. Also, it has been showed that synthetic cannabinoids could affect endocrine system [7]. AKB48, one of the synthetic cannabinoids, was first described in herbal blends in 2012. AKB48, N- (1-adamantyl)-1-pentyl-1H-indazole-3-carboxamidine, is a third-generation synthetic cannabinoids derivative also known as APINACA [8]. Because of inadequate clinical and toxicity data and potentially harmful effects of AKB48, AKB48 has not been forbidden yet, to prevent abuse and the monitoring by the World Health Organization (WHO) has been in progress [9].

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Besides possible toxicity, recent studies have focused on possible anti-cancer effects of endogenous and/or synthetic cannabinoids through endocannabinoid signaling, especially for hormone-related cancers such as breast and prostate cancer, as reviewed [10, 11]. It has been also revealed that functions of the cannabinoid and endocrine systems, especially sex hormones, were related each other by affecting stimulation and secretion of hormones [12]. There are only a few studies on the toxicity mechanisms of AKB48 including apoptosis, oxidative stress and inflammatory responses [13, 14, 15]. Therefore, we aimed to examine the relationships between the synthetic cannabinoid-AKB48 and epigenetic modifications in PC-3 cells for the first time. Because of the potential effects of synthetic cannabinoids on endocrine system, prostate cancer cell line (PC-3) was selected for present study.

2. RESULTS

2.1. Effects of AKB48 on cell viability in PC-3 cells

Effects of 7.81-1000 µM concentrations of AKB48 on cell viability in PC-3 cells assessed by MTT test. IC_{50} value of AKB48 was calculated as 343.8 µM for 24 h. As shown in Figure 1, exposure to AKB48 at the concentrations of ≥125 µM decreased the cell viability (≥27.79%) in PC-3 cells.

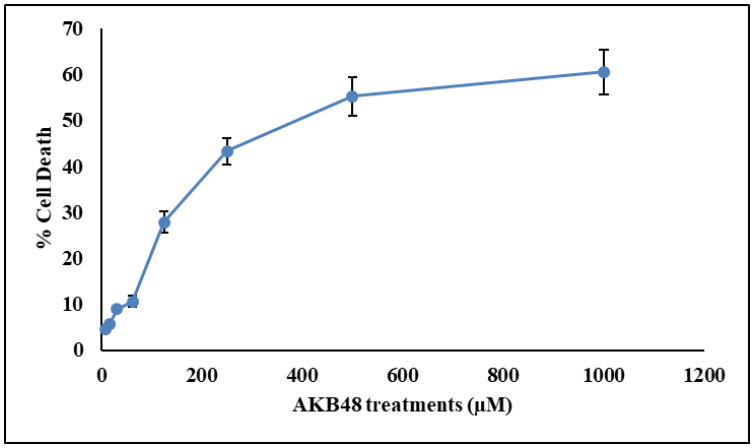

Figure 1. Effects of AKB48 (7.81-1000 µM) on cell viability by MTT test in PC-3 cells after 24 h exposure. Data are presented as mean ± SD.

2.2. Effects of AKB48 on global DNA methylation in PC-3 cells

Exposure to AKB48 resulted in significant decrease by 27% at 100 µM concentration ($p<0.05$), while no changes were observed at 10 and 50 µM concentrations, when comparing with control group (Figure 2). We found significant decrease on the expression levels of *DNMT1* (46%, $p<0.05$) (Figure 3a) and *MGMT* (66%, $p<0.05$) (Figure 3b) after 100 µM of AKB48 exposure and no changes were seen at 10 and 50 µM.

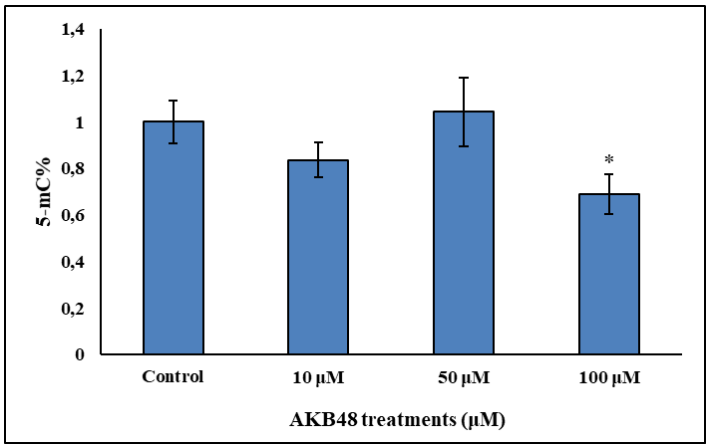

Figure 2. Effects of AKB48 (0-100 µM) on levels of 5-mC% in PC-3 cells after 24 h exposure. Data are presented as mean ± SD. Statistically significant changes are indicated by *$p<0.05$ (one way ANOVA-Dunnett post hoc test).

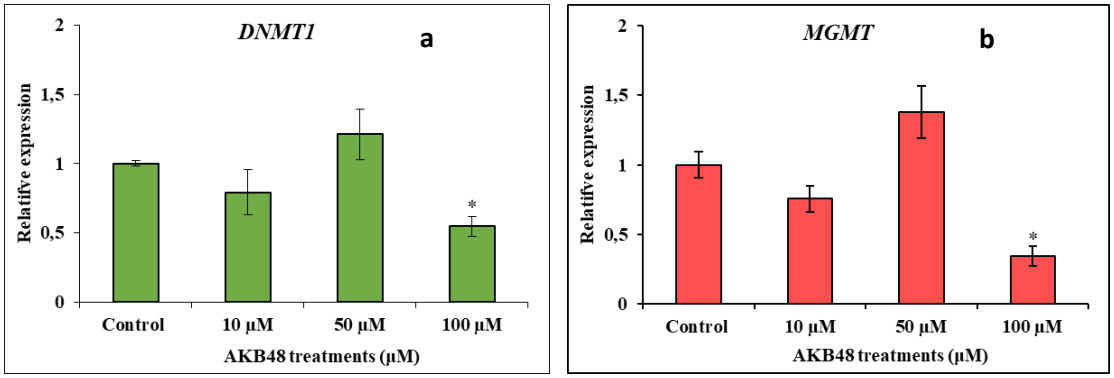

Figure 3. Effects of AKB48 (0-100 µM) on relative gene expression levels (*DNMT1* (a) and *MGMT* (b)) in PC-3 cells after 24 h exposure. Data are presented as mean ± SD. Statistically significant changes are indicated by *p<0.05 (one way ANOVA-Dunnett post hoc test).

2.3. Effects of AKB48 on gene specific DNA methylation

Gene-specific methylation analyzes in the promoter regions of the *CB1* and *CB2* genes were performed by PCR following bisulfite modification. In Figure 4, we observed that AKB48 caused partial methylation in the promoter regions of the *CB1* gene compared to the control group, while expression level of *CB1* gene was not changed. Interestingly, no data were observed regarding to the promoter-site DNA methylation and expression of *CB2* gene, in which we observed *CB2* gene was not expressed in PC-3 cells.

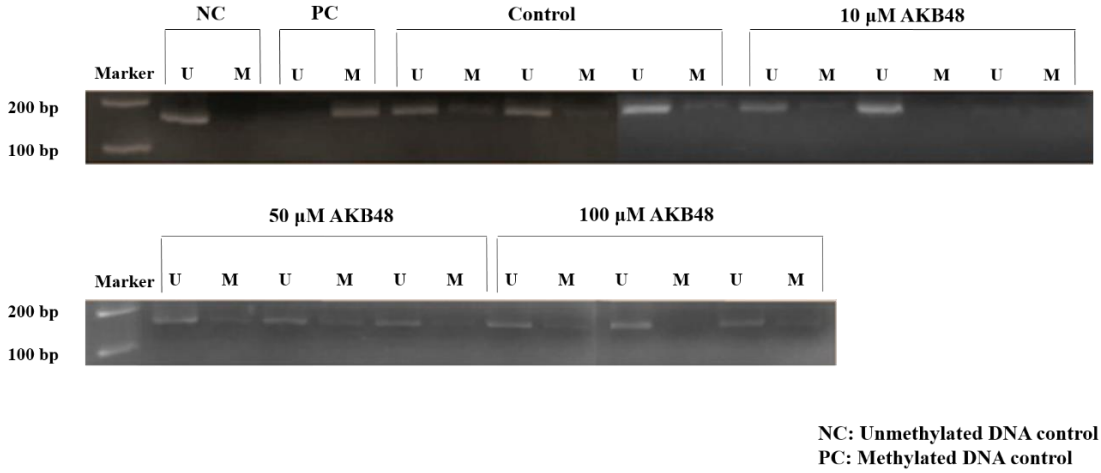

Figure 4. Effects of AKB48 (0-100 µM) on gene-specific methylation status of *CB1* in PC-3 cells after 24 h exposure. A representative sample of PC-3 cells treated with AKB48 at the concentrations of 10, 50 and 100 µM for 24 h is shown. Methylation was determined by bisulfite modification of the genomic DNA and MSP using primers for the U or M promoter sequence. NC is unmethylated DNA control and PC is methylated DNA control, as control instead of AKB48 treatment.

3. DISCUSSION

Usage of synthetic cannabinoids marketed as "Spice" or "K2" has become popular among young adults all over the world. Synthetic cannabinoids are agonists for CB1 and CB2 due to their pharmacological similarity to THC, the major psychoactive constituent in marijuana. However, synthetic cannabinoids and also their metabolites exhibit higher binding affinity at both receptors than THC and because of this more potent effect, they may have greater toxicities [16].

Limited studies have been evaluated on the cytotoxic potential of AKB48. Oztaş et al. (2019) evaluated the cytotoxicity of AKB48 in human neuroblastoma cell line (SH-SY5Y) by MTT test for 24 h and IC_{50} value of AKB48 was determined as 160.91 µM [15]. In present study, IC_{50} was found as 343.8 µM and these differences between IC_{50} values could be sourced from cell lines. Cytotoxicities of the synthetic cannabinoids, JWH-015 and R(+)-Methanandamide, were evaluated in a number of human prostate carcinoma cell lines

including LNCaP, PC-3 and DU-145 by MTT and cell viability decreased in these cells after exposure (<50 µM) for 48 h [17]. Louka et al. (2020) investigated to assess apoptosis and effects on cell cycle that the synthetic cannabinoids AM-251 and AM-1241 decreased viable cell number in DU145 and PC-3 prostate cancer cells for 48 h [18]. Several studies have shown that numerous synthetic cannabinoids caused cell death through cellular membrane damage and induction of apoptosis and also, these compounds promoted cell cycle arrest, the production of reactive oxygen species, caspases activation, disruption of the mitochondrial membrane potential *in vitro* [13, 14].

In recent years, the relationship between epigenetic modifications and substance addiction has become an increasingly remarkable field. Epigenetic mechanisms regulated the endocannabinoid system directly [19] and also indirectly by environmetal factors including alcohol, diet, stress, smoking, exercise, or drugs [20, 21]. Some studies have shown that cannabis and THC caused alterations in epigenetic regulations. It has been observed that cannabis induced elevations promoter-site CpG DNA methylation of *CB1* in peripheral blood cells of schizophrenic cannabis users [22]. Besides, it has been shown that THC altered DNA methylation [23] and resulted in histone modifications [24] and alterations in miRNA profile [23, 25]. Also, it has been shown that one of the synthetic cannabinoids, JWH-133, increased H3K4me3 levels in mouse spermatogonia [26] and altered DNA methylation levels at imprinted genes in mouse with paternal exposure [27], while WIN55,212-2 upregulated *DNMT*s and induced DNA methylation in prefrontal cortex of adolescence rats [28]. It has been known that *DNMT1* was responsible for the maintenance DNA methylation regenerating the methyl-cytosine marks. In present study, it has been found that AKB48 caused global hypomethylation which is correlated with the result of *DNMT1* gene expression level, reduced at 100 µM of AKB48 in PC-3 cells. Moreover, *CB1* gene expression did not change, while DNA methylation levels of *CB1* promoter region slightly altered after AKB48 exposure.

On the other hand, recent studies offer that cannabinoids could control cell proliferation and that importance of the endocannabinoid system emerged as potential therapeutic targets for different types of cancer growth by regulating apoptosis, proliferation, migration, adhesion, and invasion [10]. Notarnicola et al. (2008) showed that 17β-estradiol could increase and/or regulate the expressions of *CB1* and *CB2 in vitro* [29]. In addition, Purohit et al. (1980) observed that cannabinol, marihuana, and THC could interfere with androgen receptor (AR) signaling and block dihydrotestosterone binding to ARs [30]. And, these pathways are potentialy important for hormone-dependent cancers such as breast and prostate [30]. Olea-Herrero et al. (2009) have shown that phyto- and synthetic cannabinoids could be promising tools for cancer treatments because of decreased the proliferation, the invasion and angiogenesis of cancer cells and antitumour effects in prostate carcinoma cells *in vitro* [17].

O6-methylguanine-DNA methyltransferase (MGMT) improves one of the most mutagenic alkylations at the O6-position of guanine nucleotide for inhibition of cancer progression [31]. It has been shown the relation between *MGMT* gene expresion and prostate cancer and also prostate tumor biopsies had overexpression of *MGMT* [32]. In present study, it has been also observed that *MGMT* gene expression levels decreased significantly after AKB48 exposure.

4. CONCLUSION

In conclusion, the need for improving detection and recognition of synthetic cannabinoids is emerging day by day, since they are not detectable by traditional drug screenings. Besides, their potential antitumor activities, it is still very important to elucidate their potential dangers for human health, given the clinical reports on the potential psychostimulant effects of synthetic cannabinoids. The present data suggest, for the first time, that AKB48 could induce some toxic effects via epigenetic modifications. Thus, it is thought that expanding this knowledge and elucidating the causal molecular mechanisms could contribute to provide information for risk assessments and improve community welfare.

5. MATERIALS AND METHODS

5.1. Chemicals

AKB48 (AKB-1631-10) was obtained from Lipomed AG (Arlesheim, Switzerland) with informing of the Turkish Ministry of Health, Republic of Turkey. A 100 mM solution of AKB48 was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, Missouri, USA) and held at -20°C. Cell culture mediums and supplements were obtained from Wisent Bioproducts (Saint-JeanBaptiste, QC, Canada) and sterile plastic consumables were obtained from Nest (Jiangsu, China). Kits for the isolation of DNA and RNA, cDNA

synthesis and syber green master mix were purchased from Roche Life Sciences (Penzberg, Upper Bavaria, Germany). For the measurement of global DNA methylation (5-mC), elisa kit was purchased from Zymo Research (Irvine, CA, USA), Primers for gene expressions and gene-specific methylation analysis were synthesized by Sentromer DNA Technologies (Istanbul, Turkey).

5.2. Cell culture and treatments

The human prostate adenocarcinoma cell line PC-3 (ATCC® CRL-1435™) was obtained from American Type Culture Collection. PC-3 cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F-12) medium with supplements of 10% fetal bovine serum and penicillin-streptomycin (100 U-100 µg/mL). Trypsinization was used for the subculturing once the cells reached 70-80% confluence.

For gene expressions and DNA methylation analysis: prior to treatment 1×10^6 cells were maintained in a 25 cm^2 culture flask for 24 h. In the present study, we selected 10, 50, 100 µM exposure concentrations of AKB48 based on the previous study by Oztas et al. (2019) [15] and our cytotoxicity results and DMSO (0.5%) was used for the solvent control. PC-3 cells were exposed for 24 h. After trypsinization, cell were counted with trypan blue staining by using a Luna cell counter (Virginia, USA). The experiments were done in triplicates for all exposure concentrations and each experiment was repeated twice.

5.3. Cell viability assay

The cytotoxicity test of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole], was used to determine the effects of AKB48 on cell viability (33, 34). 1×10^4 cells in 100 µL medium were seeded in 96-well plates, incubated overnight for cell attachment. In the concentration range of 7.81-1000 µM of AKB48 were applied to the each well for 24 h. DMSO (0.5%) and SDS (1%) were used as solvent and positive controls, respectively and incubated for 24 h. Then, MTT test was performed and half-maximal inhibition of enzyme acvitity defined as IC_{50} value was calculated for AKB48.

5.4. Global DNA methylation analysis

The High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) was used to isolate genomic DNA from PC-3 cells according to instructions of the manufacturer. The percent of global DNA methylation was determined using the 5-mC DNA elisa kit (Zymo Research, Irvine, CA, USA) regarding to the instructions by manufacturer by using anti-5-mC monoclonal and HRP-conjugated secondary antibodies. Standard curve was prepared with the mixture of the negative (100 ng/L) and positive control (100 ng/L) standards to measure the percentage of 5-mC.

5.5. cDNA synthesis and gene expression analysis

For the expression analysis of High Pure RNA Isolation Kit (Roche Life Science) was used to isolate total RNA from cell cultures. 500 ng of total RNA was performed to synthesize first-strand cDNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science) kit with the combination of anchored-oligo(dT) and random hexamer primers and kept at -20°C. For DNA methyltransferase 1 (*DNMT1*) and O-6-methylguanine-DNA methyltransferase (*MGMT*) real-time PCR reactions were maintained by LightCycler®480 SybrGreen Master Kit (Roche, Mannheim, Germany) as described previously [35]. The primer sequences and their annealing temperatures were provided in Table 1. For the expresion analysis of *CB1* and *CB2* reseptor genes, LightCycler®480 Probes Master with RealTime Ready Custom Single Assays (Universal ProbeLibrary Probes, Roche Life Science) with target-specific primers were used in real-time quantitavie PCR. The relative expressions for all reactions were examined by the comparative Ct method and *β-actin* was selected as the reference gene [35].

5.6. Methylation-specific polymerase chain reaction (MSP)

For gene-specific DNA methylation analysis, the bisulfite conversion of genomic DNA was accomplished with EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) regarding to the instructions by manufacturer. Genomic DNA (1 µg) from control and AKB48 treatment groups was converted with CT conversion reagent for bisulfite treatment. Then, bisulfite modified DNA was eluted and stored at -80°C.

For MSP analysis, bisulfite modified unmethylated and methylated primer sequences of *CB1* and *CB2* reseptor genes were designed by using MethPrimer-Design Software [36] (Table 1). Experimental and data analysis were done as our previous study described in Demirel et al. (2015) [37].

Table 1. Primers used MSP and qPCR analysis and the corresponding annealing temperatures.

	Gene	Sense (5′-3′)	Antisense (5′-3′)	Ta (°C)	Ref.
qPCR	*DNMT1*	CCTCCAAAAACCCAGCC AAC	TCCAGGACCCTGGGGATTTC	60	[38]
	MGMT	TGCACAGCCTGGCTGAA TG	GGTGAACGACTCTTGCTGGAA	58	[39]
	β-Actin	AACTACCTTCAACTCCAT	TGATCTTGATCTTCATTGTG	48	[40]
MSP	*CB1* (M)	GGAGTATAGCGAGGTTAT GGTAGC	TAACCCCAAACTTTACTTAAAA CGA	59	[36]
	CB1 (U)	GAGTATAGTGAGGTTATG GTAGTGG	AACCCCAAACTTTACTTAAAAC AAA	58	[36]
	CB2 (M)	GATTTCGGTTTTTTGTAAT TTTC	ACTCAACCTAACGTAATAACTC ACG	57	[36]
	CB2 (U)	GATTTTGGTTTTTTGTAAT TTTTGT	TCAACCTAACATAATAACTCAC ACC	57	[36]

5.7. Statistical analysis

Results of cytotoxicity and 5-mC% were represented as mean ± standard deviation (SD). ANOVA followed by Dunnett's multiple comparison test was used for statistical analysis by using "SPSS version 21.0 for Windows" statistical program (IBM Analytics, New York, USA). For level of significance P value less than 0.05 were selected.

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