Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.3, No.2, 55-61, 2016

Letter

HU-210, a synthetic analog of Δ^9-THC, is not a modifier of estrogen signaling in MCF-7 human breast cancer cells

Hiroyuki Okazaki[1,*], Shuso Takeda[1,2,*], Hiroyuki Ishii[1], Saki Matsuo[1], Erika Furuta[1], Kazuhito Watanabe[3] and Hironori Aramaki[1,4]

[1]Department of Molecular Biology, Daiichi University of Pharmacy, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan
[2]Laboratory of Xenobiotic Metabolism and Environmental Toxicology, Faculty of Pharmaceutical Sciences, Hiroshima International University (HIU), 5-1-1 Hiro-koshingai, Kure, Hiroshima 737-0112, Japan
[3]Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920-1181, Japan
[4]Drug Innovation Research Center, Daiichi University of Pharmacy, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan

(Received February 19, 2016; Accepted February 21, 2016)

ABSTRACT — Δ^9-Tetrahydrocannabinol (Δ^9-THC), an active ingredient of marijuana, evokes a number of biological effects including anti-cancer and anti-estrogenic actions. We and others have so far focused on and investigated the latter action. We recently reported that Δ^9-THC up-regulates the expression of estrogen receptor β (ERβ, ESR2), resulting in the abrogation of 17β-estradiol (E2)-mediated ERα signaling (Takeda *et al*., Chem. Res. Toxicol., 26, 1073-1079, 2013). This finding may shed light on the possible endocrine-disrupting mechanism(s) employed by cannabinoids including Δ^9-THC. Although previous studies have suggested that HU-210, a synthetic analog of Δ^9-THC, evokes a set of endocrine alterations closely related to those of Δ^9-THC, none have examined the effects of cannabinoids with a focus on the expression of ERβ, a "suppressive" molecule for ERα-mediated signaling. Thus, we herein determined whether HU-210 is also an endocrine modifier similar to Δ^9-THC using ERα-positive MCF-7 cells in which the expression of ERβ is maintained at very low levels. The results of the present study revealed that HU-210, despite having a similar structure to Δ^9-THC, did not modulate E2/ERα signaling or induce ERβ.

Key words: HU-210, Δ^9-THC, Cannabinoids, MCF-7 cells, Estrogen receptor β, ERβ

INTRODUCTION

Estrogen receptor α (ERα, ESR1) was initially cloned approximately 30 years ago (Green *et al*., 1986). ERβ (ESR2), a second form of the ER receptor, has since been discovered and characterized (Kuiper *et al*., 1996; Weihua *et al*., 2000; Zhao *et al*., 2003, 2007). ERα and ERβ are members of the nuclear receptor family and estrogen [such as 17β-estradiol (E2)]-dependent transcription factors (Shanle and Xu, 2011). Although ERs exist in two forms, they have opposing biological role(s) in the modulation of E2 actions; ERα stimulates, whereas ERβ abrogates E2-dependent cell proliferation/migration/invasion (Weihua *et al*., 2000; Zhao *et al*., 2003, 2007). Experimental evidence suggests that the ERα/ERβ ratio is higher in malignant breast tissues (Leygue *et al*., 1998; Saji *et al*., 2000; Roger *et al*., 2001). Consistent with this finding, ERβ expression levels were found to be lower in breast cancer tissues, thereby implying that ERβ plays a suppressive role in tumorigenesis (Leygue *et al*., 1998; Roger *et al*., 2001). Thus, the expression levels of ERβ are one of the key determinants for cellular responses mediated by E2/ERα. Some (environmental) chemicals, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), exert endocrine-disrupting effects, such as anti-estrogenic actions, in animals and humans (*in vivo*) as well as in *in vitro* cell systems without directly interacting with ERα (Ruh *et al*., 1997; Cheng *et al*., 2012; Takeda *et al*., 2013; Takeda,

Correspondence: Hironori Aramaki (E-mail: haramaki@daiichi-cps.ac.jp)
*These two authors contributed equally to this work (Co-first authors).

2014). Therefore, modulators that affect the expression of ERβ may be endocrine disrupters.

Marijuana (also known as *Cannabis*) is a popular drug of abuse involving Δ⁹-THC, a biologically active component that is best recognized for its psychotropic actions. Among the biological activities of Δ⁹-THC, its accepted endocrine-disrupting effects, such as "anti-estrogenic activity", have been the main topic of research for a long period of time (Nir *et al*., 1973; Smith and Asch, 1984; Brown and Dabs, 2002; Morgan *et al*., 2012). We previously reported that the anti-estrogenic activities of Δ⁹-THC may be mediated through the up-regulation of ERβ by the cannabinoid itself (Takeda *et al*., 2013; Takeda, 2014). However, we are currently unable to conclude whether the function of Δ⁹-THC also applies to other cannabinoids.

HU-210, a synthetic cannabinoid (Mechoulam *et al*., 1990) found in the herbal mixture 'Spice' products, is an active ingredient that functions as a full agonist for cannabinoid receptor types 1 and 2 (CB1 and CB2 receptors). An examination of the structure of HU-210 revealed that this synthetic cannabinoid has a similar structure to that of natural Δ⁹-THC from the cannabis plant, although some parts differ (*See* Fig. 1). HU-210, which is structurally and pharmacologically similar to Δ⁹-THC, is currently listed as a Schedule I controlled substance under the Controlled Substances Act, as well as marijuana (Δ⁹-THC), in the United States. In contrast to Δ⁹-THC, few studies have examined the biological activities of HU-210, such as endocrine disruption (Martín-Calderón *et al*., 1998).

Based on the above-described background, we herein investigated the effects of HU-210 together with Δ⁹-THC, as a positive control, on E2/ERα-mediated signaling using a MCF-7 human breast cancer cell line as a model that abundantly expresses ERα and a very low level of ERβ (induced).

MATERIALS AND METHODS

Materials and cell cultures

HU-210 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). E2 was purchased from Nacalai Tesque (Kyoto, Japan). Δ⁹-THC was isolated and purified from drug-type cannabis leaves using established methods described elsewhere (Takeda *et al.*, 2008). The purity of Δ⁹-THC was found to be at least greater than 98% by

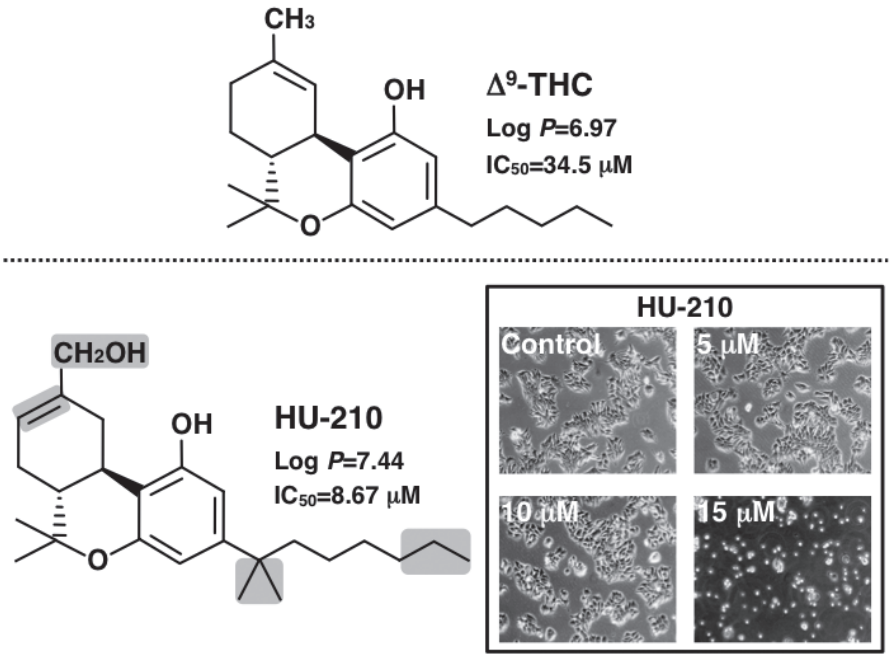

Fig. 1. Comparison of biological activities of Δ⁹-THC and its synthetic analog, HU-210. The structures of Δ⁹-THC and HU-210 are shown. In the structure of HU-210, moieties that differ from those in natural Δ⁹-THC are indicated by gray inclusions. Physical (Log *P* value) and biological (IC_{50} value) parameters for the two cannabinoids are also indicated; the Log *P* values of Δ⁹-THC and HU-210 were taken from references (Thomas *et al*., 1990; TOXNET: Toxicology Data Network). The IC_{50} value and a morphological image 48 hr after exposure to HU-210 in MCF-7 cells were specified together with the reported IC_{50} value of Δ⁹-THC (Takeda *et al*., 2013).

gas chromatography (Takeda *et al.*, 2008). All other reagents were of analytical grade, commercially available, and used without further purification. Cell culture conditions and methods were performed basically as described previously (Takeda *et al*., 2013; Okazaki *et al.*, 2015). In brief, the human breast cancer cell line, MCF-7 (obtained from the American Type Culture Collection, Rockville, MD, USA), was routinely grown in phenol red-containing minimum essential medium α (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 5% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin in a humidified incubator, within an atmosphere of 5% CO_2 at 37°C. Twenty-four hours before the chemical treatments, medium was changed to phenol red-free minimum essential medium α (MEMα, Invitrogen) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cultures of approximately subconfluence (close to 60% confluence) in a 100-mm Petri dish were used to seed for further experiments on transfection (dual-luciferase assay) and mRNA/protein expression analyses. All of the insecticides tested were prepared in dimethyl sulfoxide (DMSO, cell culture grade). In the viability experiment, cells were seeded on 96-well plates at a density of ~5,000 cells/well, and cannabinoids were introduced 4 hr after plating. After a 48-hr incubation, cell viability was analyzed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS reagent; Promega, Madison, WI, USA), according to the manufacturer's instructions. Control incubations contained the equivalent addition of DMSO (for HU-210) or ethanol (for Δ^9-THC). No significant differences were observed in cell viability or the cannabinoid effects of these solvents.

Cell morphology studies

Cell morphology studies were basically performed as described previously (Takeda *et al*., 2012). Briefly, for the morphological examination of MCF-7 cells, images were obtained using a Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany), and captured with a Pixera® Penguin 600CL Cooled CCD digital camera (Pixera Co., Los Gatos, CA, USA). Data were processed using Pixera Viewfinder 3.0 software (Pixera Co.). Breast cancer cells were plated on 6-well plates. Three areas with approximately equal cell densities were identified in each well and images of each of these areas were captured.

Transfection and luciferase reporter assay (dual-luciferase assay)

Twenty-four hours prior to transfection, MCF-7 cells (ERα-positive) were seeded (5×10^4 cells/well) on 24-well plates containing MEMα. The transfection of each expression plasmid was performed using Lipofectamine® LTX with PLUS™ reagent (Invitrogen) according to the manufacturer's instructions. DNA mixtures containing 300 ng of the (estrogen-responsive element, $ERE)_3$-Luc plasmid were co-transfected with 2 ng of the *Renilla* luciferase reporter plasmid (pRL-CMV) in the plates. Cells were washed with phosphate-buffered saline 24 hr post-transfection and changed to phenol red-free MEMα supplemented with 5% dextran-coated charcoal-treated serum, followed by the respective chemical treatments being tested. Cell extracts were then prepared using 100 µL of passive lysis buffer (Promega, Madison, WI, USA), and 20 µL was then applied to the firefly luciferase and *Renilla* luciferase assays (Dual-Luciferase Reporter Assay System, Promega) using the GloMax®-Multi Detection System (Promega). The ratio of firefly luciferase activity (expressed from reporter plasmids) to *Renilla* luciferase activity (expressed from pRL-CMV) in each sample served as a measure of normalized luciferase activity.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) analysis

Total RNA was prepared from MCF-7 cells using the RNeasy kit (Qiagen, Inc., Hilden, Germany) and purified by RNeasy/QIAamp columns (Qiagen, Inc.). In the real-time RT-PCR analysis on CDC2, ERβ, and β-actin, cDNA was prepared via RT of total RNA using the ReverTra Ace® qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). Real-time quantitative RT-PCR assays were performed with FastStart Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) and LightCycler® Nano (Roche Diagnostics, Mannheim, Germany). The primers for PCR on human CDC2 and β-actin were taken from previous studies (Takeda *et al*., 2013). The primers used for ERβ were: ERβ (sense) 5'-TTC AAA GAG GGA TGC TCA CTTC-3' and ERβ (antisense) 5'-CCT TCA CAC GAC CAG ACT CC-3'. The reaction conditions for all mRNA were 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, at 58°C for 10 sec, and 72°C for 15 sec. CDC2 and ERβ mRNA levels were normalized to the corresponding β-actin mRNA levels.

Antibodies and Western immunoblot analysis

Antibodies specific for ERβ (ab3576; Abcam, Cambridge, MA, USA) and Actin (sc-1616 HRP; Santa

Cruz Biotechnology, Santa Cruz, CA, USA) were used. Whole cell extracts were prepared using CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, Dorset, UK) supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Hudson, NH, USA). SDS-polyacrylamide gel electrophoresis/Western immunoblotting was performed based on procedures described previously (Okazaki *et al*., 2014). The membranes were photographed using the Chemi Doc XRS plus system (Bio-Rad, Hercules, CA, USA). Equal amounts of protein for each sample were confirmed by probing with Actin.

Data analysis

Differences were considered significant when the *P* value was calculated to be less than 0.05. A data analysis of differences among multiple groups was performed using Dunnett's test. Calculations were performed using Statview 5.0J software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

We first explored the effects of HU-210 on the morphology and viability of MCF-7 cells. As shown in Fig. 1A (lower panel), MCF-7 cells exhibited a rounder morphology after a 48-hr exposure in a concentration-dependent manner up to 15 μM than the vehicle-treated control; the IC_{50} value was determined to be 8.67 μM. Almost complete cell death was observed at 25 μM (data not shown). As we reported previously, the inhibitory efficacy of HU-210 on cell viability was more potent than that of Δ^9-THC (*i.e.*, IC_{50} = 34.5 μM) (Takeda *et al*., 2013). In this study, Δ^9-THC was utilized as a positive control in order to analyze the anti-estrogenic potential of HU-210 for E2/ERα signaling (Takeda *et al*., 2013; Takeda, 2014). Based on their IC_{50} values, we subsequently investigated the effects of the two cannabinoids on E2-driven ERα activation. Physiological levels of E2 (1 nM) activated the ERα activity around 10-fold compared to vehicle-treated controls (Fig. 2B). Even though Δ^9-THC abrogated E2/ERα activation in a concentration-dependent manner under these conditions, HU-210 was inactive, even at 10 μM (> IC_{50} value) (Figs. 2A and 2B). A comparison between Δ^9-THC and HU-210 at concentrations of 5 and 10 μM revealed that the former significantly inhibited transcriptional activities, whereas the latter did not. Consistent with the results of ERα activation analyses, the expression levels of CDC2, a gene regulated by ERα (Lin *et al*., 2007; Takeda *et al*., 2013), were not affected by HU-210 even at 10 μM (Figs. 3A and 3B), while Δ^9-THC abrogated its expression even in experiments using concentrations less than the IC_{50} value (*i.e.*, 25 μM versus 34.5 μM) (Fig. 3A, left panel) (Takeda *et al*., 2013). When we focused on the expression status of ERβ, a negative modifier of E2-mediated ERα signaling (Weihua *et al.*, 2000; Zhao *et al*., 2003, 2007), the cannabinoid Δ^9-THC up-regulated the expression of ERβ by approximately 3-fold that of the controls at 25 μM, whereas no observable changes

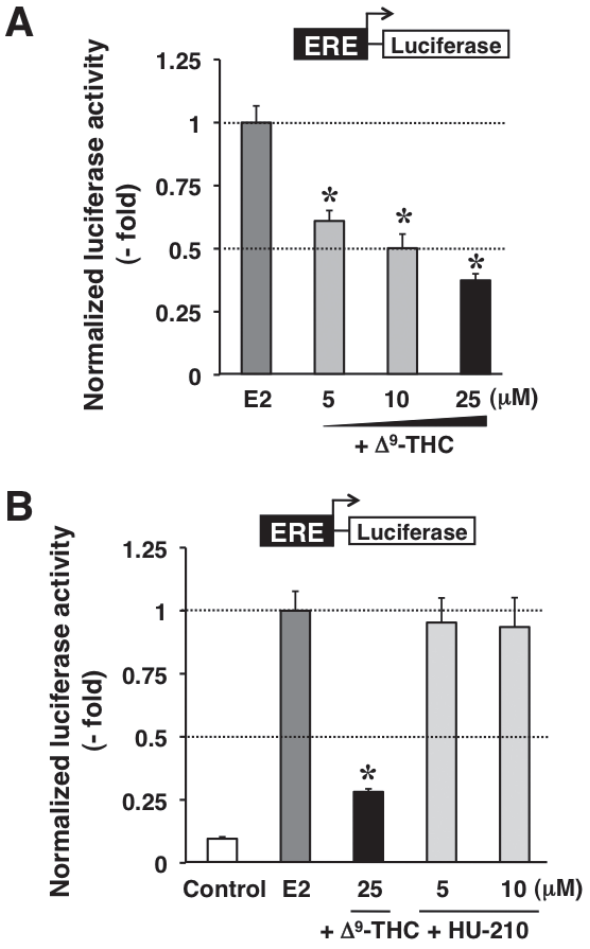

Fig. 2. Effects of Δ^9-THC and HU-210 on estrogen signaling in ERα-positive MCF-7 cells. MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, MCF-7 cells were treated with Δ^9-THC (5, 10, or 25 μM) (A) and HU-210 (5 or 10 μM) (B) in the presence of 1 nM E2. In the Control incubation, cells were treated with vehicle alone. After 24 hr, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as a fold induction from the E2-treated group (indicated as 1), as the mean ± S.E. (n = 3). *Significantly different ($P < 0.05$) from the E2-treated group.

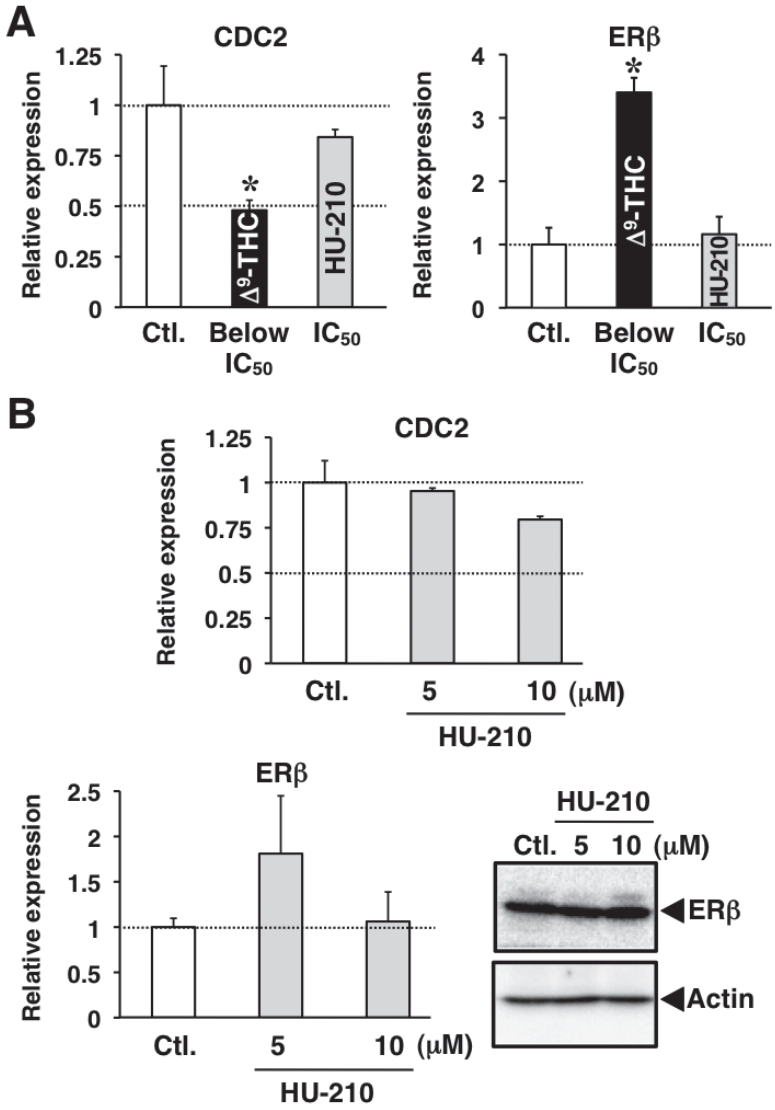

Fig. 3. Effects of Δ⁹-THC and HU-210 on CDC2 and ERβ expression in MCF-7 cells. (A) Real-time RT-PCR analyses of CDC2 (left panel) and ERβ (right panel) in MCF-7 cells 48 hr after the treatment with vehicle (Ctl., Control) or Δ⁹-THC (25 μM; less than its IC_{50} value) and HU-210 (8.67 μM; its IC_{50} value). (B) Real-time RT-PCR analyses of CDC2 and ERβ in MCF-7 cells 48 hr after the treatment with vehicle (Ctl., Control) or HU-210 (5 or 10 μM). Data are expressed as a fold induction from the vehicle-treated control (Ctl.) (indicated as 1), as the mean ± S.E. (n = 3). *Significantly different ($P < 0.05$) from the vehicle-treated control. A Western immunoblot analysis of ERβ. MCF-7 cells were treated with 5 or 10 μM HU-210 or vehicle (indicated as Ctl.) for 48 hr. Total cell lysates were prepared, and Western immunoblot analyses were performed using antibodies specific for ERβ and Actin, respectively. Actin was used as an internal loading control.

were observed in its expression in HU-210-treated samples (Figs. 3A, right panel and 3B, left panel). Furthermore, this inactivity in the induction of ERβ noted in the mRNA experiments was also demonstrated in the proteins levels of ERβ (Fig. 3B, right panel).

Cannabinoids generally exert their biological effects, including anti-proliferative effects on cancer cells, via the activation of CB receptors expressed in cells (Pertwee *et al*., 2010). Although the Log *P* values of the two cannabinoids were similar, namely, highly lipophilic (Fig. 1) (Thomas *et al*., 1990; TOXNET: Toxicology Data Network), the binding affinity (*K*i) of HU-210 to the human

CB1/CB2 receptors (0.061/0.52 nM, data from Tocris Bioscience) was previously shown to be markedly higher than that of Δ^9-THC (53.3/75.3 nM) (Felder *et al.*, 1995). Thus, the potencies of IC_{50} values between these cannabinoids in the abrogation of MCF-7 cell proliferation may be attributed to differences in *K*i values for the receptors. Martín-Calderón *et al*. (1998) reported that HU-210 induced a set of endocrine alterations, such as plasma hormones including growth, luteinizing, and follicle-stimulating hormones, at doses 50-200-fold lower than those required for Δ^9-THC in adult female rats. These effects of cannabinoids appear to be triggered by their activation of CB receptors; however, since the Δ^9-THC-mediated up-regulation of ERβ was not negated by specific antagonists for CB1 and CB2 (Takeda, 2014), possible requirement(s) for the up-regulation of ERβ may not involve CB receptors. As clearly shown in Fig. 1, the structures of Δ^9-THC and HU-210 were "similar", but also different at the 4 indicated points. Although HU-210 evoked endocrine disruption in rat models, similar to Δ^9-THC (Martín-Calderón *et al*., 1998), HU-210 may be devoid of the ability to modulate the estrogen signaling responsible for up-regulating ERβ. Further studies are needed in order to ascertain this issue by focusing on their structural discrepancies.

ACKNOWLEDGMENTS

This research was supported by EXTEND2010 grants from the Ministry of the Environment, Japan (to H.A.), and in part by a Grant-in-Aid for Scientific Research (C) [25460182, (to S.T.)] from the Japan Society for the Promotion of Science (JSPS) KAKENHI.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Brown, T.T. and Dobs, A.S. (2002): Endocrine effects of marijuana. J. Clin. Pharmacol., **42** (Suppl.), 90S-96S.

Cheng, L., Li, J., Han, Y., Lin, J., Niu, C., Zhou, Z., Yuan, B., Huang, K., Li, J., Jiang, K., Zhang, H., Ding, L., Xu, X. and Ye, Q. (2012): PES1 promotes breast cancer by differentially regulating ERα and ERβ. J. Clin. Invest., **122**, 2857-2870.

Felder, C.C., Joyce, K.E., Briley, E.M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A.L. and Mitchell, R.L. (1995): Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol. Pharmacol., **48**, 443-450.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P. and Chambon, P. (1986): Human oestrogen receptor cDNA: sequence, expression and homology to *v-erb-A*. Nature, **320**, 134-139.

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S. and Gustafsson, J.A. (1996): Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA, **93**, 5925-5930.

Leygue, E., Dotzlaw, H., Watson, P.H. and Murphy, L.C. (1998): Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res., **58**, 3197-3201.

Lin, C.Y., Ström, A., Li Kong, S., Kietz, S., Thomsen, J.S., Tee, J.B., Vega, V.B., Miller, L.D., Smeds, J., Bergh, J., Gustafsson, J.A. and Liu, E.T. (2007): Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. Breast Cancer Res., **9**, R25.

Martín-Calderón, J.L., Muñoz, R.M., Villanúa, M.A., del Arco, I., Moreno, J.L., de Fonseca, F.R. and Navarro, M. (1998): Characterization of the acute endocrine actions of (–)-11-hydroxy-Δ^9-tetrahydrocannabinol-dimethylheptyl (HU-210), a potent synthetic cannabinoid in rats. Eur. J. Pharmacol., **344**, 77-86.

Mechoulam, R., Lander, N., Breuer, A. and Zahalka, J. (1990): Synthesis of the individual, pharmacologically distinct, enantiomers of a tetrahydrocannabinol derivative. Tetrahedron: Asymmetry, **1**, 315-318.

Morgan, D.J., Muller, C.H., Murataeva, N.A., Davis, B.J. and Mackie, K. (2012): Δ^9-Tetrahydrocannabinol (Δ^9-THC) attenuates mouse sperm motility and male fecundity. Br. J. Pharmacol., **165**, 2575-2583.

Nir, I., Ayalon, D., Tsafriri, A., Cordova, T., Lindner, H.R. (1973): Letter: Suppression of the cyclic surge of luteinizing hormone secretion and of ovulation in the rat by Δ^1-tetrahydrocannabinol. Nature, **243**, 470-471.

Okazaki, H., Matsunaga, N., Fujioka, T., Okazaki, F., Akagawa, Y., Tsurudome, Y., Ono, M., Kuwano, M., Koyanagi, S. and Ohdo, S. (2014): Circadian regulation of mTOR by the ubiquitin pathway in renal cell carcinoma. Cancer Res., **74**, 543-551. Erratum in: Cancer Res., **74**, 1621 (2014).

Okazaki, H., Takeda, S., Ikeda, E., Fukunishi, Y., Ishii, H., Taniguchi, A., Tokuyasu, M., Himeno, T., Kakizoe, K., Matsumoto, K., Shindo, M. and Aramaki, H. (2015): Bongkrekic acid as a selective activator of the peroxisome proliferator-activated receptor γ (PPARγ) isoform. J. Toxicol. Sci., **40**, 223-233.

Pertwee, R.G., Howlett, A.C., Abood, M.E., Alexander, S.P., Di Marzo, V., Elphick, M.R., Greasley, P.J., Hansen, H.S., Kunos, G., Mackie, K., Mechoulam, R. and Ross, R.A. (2010): International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB_1 and CB_2. Pharmacol. Rev., **62**, 588-631.

Roger, P., Sahla, M.E., Mäkelä, S., Gustafsson, J.A., Baldet, P. and Rochefort, H. (2001): Decreased expression of estrogen receptor β protein in proliferative preinvasive mammary tumors. Cancer Res., **61**, 2537-2341.

Ruh, M.F., Taylor, J.A., Howlett, A.C. and Welshons, W.V. (1997): Failure of cannabinoid compounds to stimulate estrogen receptors. Biochem. Pharmacol., **53**, 35-41.

Saji, S., Jensen, E.V., Nilsson, S., Rylander, T., Warner, M. and Gustafsson, J.A. (2000). Estrogen receptors α and β in the rodent mammary gland. Proc. Natl. Acad. Sci. USA, **97**, 337-342.

Shanle, E.K. and Xu, W. (2011): Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action. Chem. Res. Toxicol., **24**, 6-19.

Smith, C.G. and Asch, R.H. (1984): Acute, short-term, and chron-

ic effects of marijuana on the female primate reproductive function. NIDA Res. Monogr., **44**, 82-96.

Takeda, S., Misawa, K., Yamamoto, I. and Watanabe, K. (2008): Cannabidiolic acid as a selective cyclooxygenase-2 inhibitory component in cannabis. Drug Metab. Dispos., **36**, 1917-1921.

Takeda, S., Okajima, S., Miyoshi, H., Yoshida, K., Okamoto, Y., Okada, T., Amamoto, T., Watanabe, K., Omiecinski, C.J. and Aramaki, H. (2012): Cannabidiolic acid, a major cannabinoid in fiber-type cannabis, is an inhibitor of MDA-MB-231 breast cancer cell migration. Toxicol. Lett., **214**, 314-319.

Takeda, S., Yoshida, K., Nishimura, H., Harada, M., Okajima, S., Miyoshi, H., Okamoto, Y., Amamoto, T., Watanabe, K., Omiecinski, C.J. and Aramaki, H. (2013): Δ^9-Tetrahydrocannabinol disrupts estrogen-signaling through up-regulation of estrogen receptor β (ERβ). Chem. Res. Toxicol., **26**, 1073-1079.

Takeda, S. (2014): Δ^9-Tetrahydrocannabinol targeting estrogen receptor signaling: the possible mechanism of action coupled with endocrine disruption. Biol. Pharm. Bull., **37**, 1435-1438.

Thomas, B.F., Compton, D.R. and Martin, B.R. (1990): Characterization of the lipophilicity of natural and synthetic analogs of Δ^9-tetrahydrocannabinol and its relationship to pharmacological potency. J. Pharmacol. Exp. Ther., **255**, 624-630.

TOXNET: Toxicology Data Network. Chemical Information Profile for HU-210 [CAS No. 112830-95-2]. Available online: http://chem.sis.nlm.nih.gov/chemidplus/rn/112830-95-2.

Weihua, Z., Saji, S., Mäkinen, S., Cheng, G., Jensen, E.V., Warner, M. and Gustafsson, J.A. (2000): Estrogen receptor (ER) β, a modulator of ERα in the uterus. Proc. Natl. Acad. Sci. USA, **97**, 5936-5941.

Zhao, C., Lam, E.W., Sunters, A., Enmark, E., De Bella, M.T., Coombes, R.C., Gustafsson, J.A. and Dahlman-Wright, K. (2003): Expression of estrogen receptor β isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. Oncogene, **22**, 7600-7606.

Zhao, C., Matthews, J., Tujague, M., Wan, J., Ström, A., Toresson, G., Lam, E.W., Cheng, G., Gustafsson, J.A., and Dahlman-Wright, K. (2007): Estrogen receptor β2 negatively regulates the transactivation of estrogen receptor α in human breast cancer cells. Cancer Res., **67**, 3955-3962.