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Veterinary drug, 17β-trenbolone promotes the proliferation of human prostate cancer cell line through the Akt/AR signaling pathway

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HIGHLIGHTS

- 17 TB is hydrolyzed form of veterinary drug, Trenbolone acetate.
- 17 TB residues in meat and milk products could cause adverse effects in humans.
- We found mechanism of proliferation of human prostate cancer cell, 22Rv1 by 17 TB.
- 17 TB promotes the proliferation of 22Rv1 line through Akt/AR signaling pathway.

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ABSTRACT

Trenbolone acetate (TBA) is a synthetic anabolic steroidal growth factor that is used for rapid muscle development in cattle. The absorbed TBA is hydrolyzed to the active form, 17β-trenbolone (17 TB; 17β-hydroxy-estra-4,9,11-trien-3-one) in meat and milk products, which can cause adverse health effects in humans. Similar to 5α-dihydrotestosterone (DHT), 17 TB was reported to exhibit endocrine disrupting effects on animals and humans due to its androgenic effect via binding to the androgen receptor. The purpose of this study is to investigate the molecular mechanism of cell proliferation in prostate cancer (PCa) cells treated with 17 TB. We found that 17 TB induces AR-dependent cell proliferation in the human prostate cancer cell line, 22Rv1 in a concentration dependent manner. Treatment with 17 TB increased the expression of cell cycle regulatory proteins, cyclin D2/CDK-4 and cyclin E/CDK-2, whereas the expression of p27 was down-regulated. Furthermore, phosphorylation of Rb and activation of E2F were also induced, which suggests the activation of cyclin D2/CDK-4 and cyclin E/CDK-2 in the cells. When 22Rv1 cells were exposed to 30 pM of 17 TB, which is the effective concentration (EC_{50}) value required to observe proliferative effects on 22Rv1 cells, the expression levels of the phosphorylated forms of Akt and GSK3β were increased. This study demonstrates that 17 TB induces AR-dependent proliferation through the modulation of cell cycle-related proteins in the Akt signaling pathway. The present study provides an effective methodology for identifying cell proliferation signaling of veterinary drugs that exert AR agonistic effects.

1. Introduction

More than 260,000 chemicals have been distributed to meet the needs of industrial growth, and over 40,000 chemicals are used in Korea (Kim et al., 2017). Despite the benefits these products afford, humans and wildlife are continuously exposed to the potentially hazardous chemicals present in them (Klepac et al., 2000). Over the last two decades, there has been international attention on environmental exposure, and the effects of endocrine disrupting chemicals (EDCs) in humans and wildlife (Bergman et al., 2013). EDCs are exogenous molecules that can mimic or inhibit the action of sexual hormone receptors such as estrogen, androgen and

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thyroid hormone receptors. These actions also disrupt the synthesis, movement, metabolism, and secretion of naturally occurring hormones and might cause obesity, diabetes and cancer (Dominik, 2015; Evanthia et al., 2009).

Livestock and aquatic animals may be exposed to growth promoters as feed additives (Arnold et al., 2013). These veterinary drugs tend to be used mostly in antibiotics and steroidal hormones. It is expected that consumption of veterinary drugs will increase due to growing agriculture and aquaculture production which must meet the food demands of an expanding human population (Bártíková et al., 2016). However, the presence of several veterinary drug residues in meat and milk products could cause potential adverse health effects on the human endocrine system (Baynes et al., 2016). For example, zeranol, a synthetic tetrahydro-derivative of zearalenone, is used as a growth promoter to accelerate weight gain, improve the feed conversion efficacy and increase the lean meat-to-fat ratio (Takashi et al., 2006). It may have endocrine-disrupting effects, because it mimics estrogen activity through binding affinity to the estrogen receptor (Takemura et al., 2007; Ye et al., 2011). In addition, the steroidal growth promoter, trenbolone acetate (TBA) is hydrolyzed to the active form, 17β-trenbolone (17 TB; 17β-hydroxy-estra-4,9,11-trien-3-one) in the body, and it exerts androgenic effects by binding androgen receptors similarly to 5α-dihydrotestosterone (DHT) (Jennifer et al., 2009; Vickie et al., 2002; Wilson et al., 2002). TBA and its metabolites have been identified as EDCs through many studies and are associated with reproductive toxicity (Vickie et al., 2002).

Prostate cancer (PCa) is one of the most commonly diagnosed cancers in man, and death rates increase every year (Ahmedin et al., 2006). The androgen receptor plays a critical role in the progression of PCa through the regulation of cell cycle-related gene expression (Michael et al., 2001). Additionally, Akt pathway signaling is frequently activated in PCa and plays an important role in the proliferation of PCa *via* phosphorylation (Liangliang and Xuesen, 2014; Rhonda and Andrew, 2013; Vijayalakshmi et al., 2011). Activated Akt cans promote cell survival by inactivating glycogen synthase kinase-3 beta (GSK-3β) (Thomas et al., 2003), which is another main Akt target gene that is critically involved in the induction of apoptosis and cell cycle arrest (Yiwei et al., 2007).

In this study, we confirmed that the proliferative effect of 17 TB on human prostate cancer cell line, 22Rv1, induced both androgen-dependent growth and prostate specific antigen (PSA) secretion (Clifford et al., 2002; Hartel et al., 2003) and the PC3 line, is one of the representative androgen-independent cell lines (Kim et al., 2006). In addition, the agonistic effect against human androgen receptor (AR) of 17 TB was also measured by a 22Rv1/MMTV AR transcriptional activation (TA) assay, which was developed in our previous study (Sun et al., 2016). Our findings indicate that 17 TB promotes proliferation of the human androgen-dependent prostate cancer cells line by modulation of the cell cycle related proteins via the Akt/AR signaling pathway.

2. Materials and methods

2.1. Cell culture

The human PCa cell lines 22Rv1 and PC3 were purchased from the American Type Culture Collection (Rockville, MD). The 22Rv1/MMTV cells were applied to determine the AR agonistic effect of 17 TB. These cells were permanently transfected with the pGL4/MMTV vector (Promega, Madison, WI), which possesses a murine mammary tumor virus (MMTV) LTR region containing an androgen response element in our previous report (Sun et al., 2016). The MMTV sequence was sub-cloned into pGL4_Hygro (Promega, Madison, USA) using Lipofectamine® 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Cells were maintained in RPMI1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 1 × antibiotic-antimycotic (Anti-Anti; Gibco, Grand Island, NY), and 2 mM of GlutaMax™ (Gibco, Grand Island, NY) in a humidified 5% CO_2 atmosphere at 37 °C. For AR TA assays, cells were cultured in phenol red-free RPMI 1640 (Gibco, Grand Island, NY) supplemented with 5% charcoal-dextran stripped FBS (CSF; PAA, Australia), 1 × Anti-Anti (Gibco, Grand Island, NY) and GlutaMAX™ (Gibco, Grand Island, NY).

2.2. Analysis of cell proliferation by MTT assay

The cell proliferative effect was analyzed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma, St. Louis, MO). The 22Rv1 cells (3×10^4 cells/well) were seeded into 96-well culture plates and were cultured in phenol red-free RPMI 1640 supplemented with 5% CSF (PAA, Australia), 1 × Anti-Anti (Gibco, Grand Island, NY), and GlutaMAX™ (Gibco, Grand Island, NY) in a 5% CO_2 atmosphere at 37 °C for 24 h. Cells were exposed to 17 TB at various concentrations for 48 h. Then, MTT reagent was added to each well and the plates were incubated for 4 h at 37 °C. Media including MTT was removed from each well and the formazan crystals were dissolved by DMSO. The absorbance was measured at 570 nm on a microplate spectrophotometer (EL808; Bio-Tek Instruments, Winooski, VT).

2.3. AR fluorescence polarization competitive binding assay

The binding affinity of 17 TB against AR was evaluated using a fluorescent probe according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Competitive binding assays were performed in 384-well black plates (Corning, Corning, NY) in triplicate. The 17 TB was prepared by performing 10-fold serial dilutions sequentially with assay buffer. Then, 17 TB and Fluormone™ AL Green were mixed with recombinant AR, and the plates were incubated in the dark at room temperature for 4 h. The fluorescent polarization values were measured in each well using 480 nm excitation and 535 nm emission interference filters from Envision (PerkinElmer, Wellesley, MA). The relative binding affinity (RBA; relative to DHT) values were calculated as follows: RBA (%) = (IC_{50} of DHT)/(IC_{50} of test chemical) × 100.

2.4. 22Rv1/MMTV AR transcriptional activation assay

The cells (3×10^4 cells/well) were seeded in 96-well culture plates (Corning, Corning, NY). After incubation for 48 h, the cells were treated with a serial dilution of 17 TB along with reference chemicals (positive control: 5α-dihydrotestosterone (DHT), negative control: diethylhexyl phthalate (DEHP)) and AR agonist (mestanolone). After incubation for 24 h, the media was removed from each well and replaced with luciferase assay solution (50 μL/well) (Steady-Glo® Luciferase Assay System, Promega, Madison, WI). After shaking for 10 min at ambient temperature, the luciferase activity was measured using a MicroBeta2 2450 plate counter (Perkin-Elmer, Waltham, MA).

2.5. Western blot analysis

Cells were washed with PBS and lysed with RIPA buffer (50 mM of Tris-Cl, 150 mM of sodium chloride, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM of EDTA) supplemented with protease inhibitor (Cell Signaling Technology, Danvers, MA). Proteins were subjected to sodium dodecyl sulfate-poly acrylamide gel