Selective Androgen Receptor Modulator, YK11, Up-Regulates Osteoblastic Proliferation and Differentiation in MC3T3-E1 Cells

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Received September 18, 2017; accepted December 11, 2017

Androgens are key regulators that play a critical role in the male reproductive system and have anabolic effects on bone mineral density and skeletal muscle mass. We have previously reported that YK11 is a novel selective androgen receptor modulator (SARM) and induces myogenic differentiation and selective gene regulation. In this study, we show that treatment of YK11 and dihydrotestosterone (DHT) accelerated cell proliferation and mineralization in MC3T3-E1 mouse osteoblast cells. Further, YK11-treated cells increased osteoblast specific differentiation markers, such as osteoprotegerin and osteocalcin, compared to untreated cells. These observations were attenuated by androgen receptor (AR) antagonist treatment. To clarify the effect of YK11, we investigated rapid non-genomic signaling by AR. The phosphorylated Akt protein level was increased by YK11 and DHT treatment, suggesting that YK11 activates Akt-signaling via non-genomic signaling of AR. Because it is known that signaling is a key regulator of androgen-mediated osteoblast differentiation, YK11 has osteogenic activity as well as androgen.

Key words androgen receptor; selective androgen receptor modulator; MC3T3-E1 cell; osteoblastic differentiation

Androgens are key steroidal hormones that play a critical role in the male reproductive system as in prostatic and testicular development. Androgens also show anabolic effects on bone mineral density and skeletal muscle mass.1–3 In men, an age-related decline in serum testosterone which is one of the androgen levels is associated with reduced muscle mass and bone density. Androgen replacement therapy improves the limitations in physical function and poor mobility in older men.4 In the United States, androgen replacement therapy is used in about one million male patients aged 40 years or older.4 However, many undesirable side effects (e.g., prostatic hyper trophy, hair loss, and hoarse) are observed in androgen replacement therapy using testosterone.5–7 Whereas, estrogen replacement therapy prevents osteoporosis, but may concurrently cause mammary and uterine hyperplasia.8–10 Currently, selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, have been used for osteoporosis11 and breast cancer.12 SERMs have beneficial effect of estrogens with little adverse side effect via estrogen receptor. Similarly, development of selective androgen receptor modulators (SARMs) is expected.13–15 SARMs have an anabolic action in bone and muscle without an androgenic action on male sex organs via the androgen receptor (AR).16–18

AR belongs to a member of the nuclear receptor (NR) superfamily of ligand-dependent transcriptional factors. In the absence of a ligand, AR is localized in the cytoplasm. Upon binding with androgens, AR translocates into the nucleus.19–23 Following nuclear translocation, the AR homodimer binds to androgen responsive elements (AREs) in the enhancer regions of its target genes. We have previously proposed a synthetic steroidal compound, (17α,20E)-17,20-[(1-methoxyethylidene)-bis(oxyl)]-3-oxo-19-norpregna-4,20-diene-21-carboxylic acid methyl ester (named YK11), as a novel candidate for SARM. This compound has a partial agonistic effect on AR compared to dihydrotestosterone (DHT) as determined by a reporter gene assay.24 Furthermore, we found that YK11 accelerates myogenic differentiation in C2C12 myoblast cells.25

The present study focus on the bone anabolic effect of YK11 on osteoblastic differentiation. The alkaline phosphatase (ALP) activity, cell growth, mineralization, and the mRNA levels of markers for osteoblastic differentiation were assessed in YK11-treated mouse osteoblastic cell line MC3T3-E1. In addition, the effect of YK11 stimulates rapid non-genomic activation of Akt signaling, which is a key regulator of androgen-mediated osteoblast differentiation was evaluated.

MATERIALS AND METHODS

Chemicals YK11 was prepared as previously reported.24 DHT, hydroxy flutamide (HF), ascorbic acid, and β-glycerophosphate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell Culture Mouse osteoblast cells, MC3T3-E1, were cultured in Minimum Essential Medium (MEM) α (Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum (FBS) (Bio-sera, MO, U.S.A.) at 37°C in a humidified atmosphere with 5% CO₂. MC3T3-E1 cells were seeded on plates and maintained in MEM α supplemented with 10% charcoal-Stripped FBS (csFBS) (Gibco) for 24 h. To induce osteoblast differentiation, YK11 or DHT in MEM α supplemented with 10% csFBS, 50 µg/mL ascorbic acid, and 5 mM β-glycerophosphate (differentiation medium) were added to the cells on day 0. The medium replacement was performed every 3 or 4 d.

MTS Cell Proliferation Assay MC3T3-E1 cells were seeded in 96-well microplates at a density of 5000 cells per well for 24 h with csFBS and were treated with solvent control, DHT, or YK11 as indicated. After 96 h of incubation, cell viability was evaluated using the MTS assay kit (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions. The number of living cells is directly proportional to the absorbance at 490 nm of a formazan product reduced from...
MTS by living cells. Absorbance at 490 nm was measured in a multimode detector (BECKMAN COULTER, CA, U.S.A.).

**Alizarin Red S Staining** Mineralization (calcium deposit (ion)) in osteoblast differentiation was investigated by alizarin red S (Wako Pure Chemical Industries, Ltd.) staining.

For alizarin red S staining, cells were fixed with cold methanol at 4°C for 20 min after rinsed three times with phosphate buffered saline (PBS), and then rinsed three times with H2O to remove methanol completely at day 10. The cells were then stained with 40 mM alizarin red S stain solution (pH 6.38) for 15 min to stain the calcium deposits. Next, the stained cells were rinsed five times with H2O to remove the unbound alizarin red S and the stained cultures were imaged.

**ALP Activity** ALP activity was measured by using Lab Assay™ ALP (Wako Pure Chemical Industries, Ltd.). On the indicated experimental endpoints, after the medium was removed, the cell layers were rinsed thrice with PBS, and lysed by 0.05% Triton X-100 (MP Biomedicals, LLC, France). The cell lysates were mixed with 0.1 M carbonate buffer (pH 9.8) containing 2 mM MgCl2 and 6.7 mM of p-nitrophenyl phosphate. The reaction mixture was incubated at 37°C for 15 min and the reaction was stopped by adding NaOH. Absorbance was measured at 405 nm in a multimode detector (BECKMAN COULTER).

**Real-Time RT-Quantitative (q)PCR** Total RNA was isolated using ISOGEN II (Nippon Gene, Toyama, Japan). The cDNA was synthesized using the ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time qPCR was conducted using KOD SYBR qPCR Mix (TOYOBO) in a final volume of 25 μL according to the manufacturer’s protocol, and the results were analyzed using Applied Biosystems 7500 Fast System SDS software. The primer pairs used are shown in Table 1.

**Immunoblotting** Cells were harvested and lysed in sodium dodecyl sulfate (SDS) sample buffer containing 125 mM Tris–HCl, pH 6.8, 4% SDS, 10% sucrose, 10 mM dithiothreitol, and 0.01% bromophenol blue. Whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting was performed using anti-Akt (Biologend, CA, U.S.A.), anti-phospho Ser473 Akt (p-Akt) (Cell Signaling Technology, MA, U.S.A.), and horseradish peroxidase-conjugated anti-tubulin antibodies (MBL, Nagoya, Japan) as primary antibodies. Horseradish peroxidase-conjugated anti-mouse or rabbit immunoglobulin G (IgG) antibody (Cell Signaling Technology, MA, U.S.A.) was used as the secondary antibody. Band density was quantitated using Image J software (Image J 1.43, National Institute of Mental Health, NA, U.S.A.)

**Statistical Analysis** Statistically significant differences were determined using one-way ANOVA followed by Dunnett’s multiple comparison as the post hoc test, and differences were considered statistically significant at p<0.05.

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### Table 1. Primers for the Target Genes (5’→3’)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (Fw)</th>
<th>Reverse (Rev)</th>
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<tbody>
<tr>
<td>Osteoprotegerin (OPG)</td>
<td>TGAGAGAACGAGAAAGACCTGC</td>
<td>CGGATTGAACCTGATTCCCTAT</td>
</tr>
<tr>
<td>Osteocalcin (OC)</td>
<td>CGGCCCTGAGCTGACAAAA</td>
<td>GCCGGAGTCGTTCTACCTACT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TCCCTCTGAGCAGAAGTACTC</td>
<td>CTGCTTGCTGATCCACATC</td>
</tr>
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**RESULTS**

**Effect of YK11 on Osteoblast Proliferation** The first step of osteoblast differentiation is known to be cell proliferation. Thus, to investigate whether YK11 accelerates osteoblast cell growth, we performed the MTS assay. Osteoblastic MC3T3-E1 cells were treated with YK11 0.5 μM and DHT 0.01 μM for 96 h. The result showed that the cell growth were increased by YK11 and DHT treatments. Further, these observations were reversed by co-treatment with the AR antagonist, HF (Fig. 1). These results indicate that YK11 accelerates osteoblast cell proliferation via AR similar to DHT.

**Effect of YK11 on Osteoblast ALP Activity and Mineralization** Increase in the activity of the membrane bound ecto-enzyme ALP is a key indicator of early stage in osteoblast differentiation. MC3T3-E1 cells were cultured in differentiation medium and treated with YK11 or DHT for 10 d. ALP activity of YK11 and DHT-treated cells increased as compared to that in solvent-treated cells (Fig. 2A). These increases in ALP activity were inhibited by co-treatment with HF (Fig. 2A).

We further investigated mineralization in mature osteoblasts using alizarin red S staining. MC3T3-E1 cells were cultured in differentiation medium and treated with YK11 or DHT for 21 d. Calcium deposits were observed in YK11 and DHT treated cells but not in solvent-treated cells (Fig. 2B). Taken together, these observations suggest that YK11 promotes osteoblast differentiation with mineralization and increased ALP activity and that these effects are produced by AR similar to DHT.

**Effect of YK11 on the Expression of Osteogenic Markers** Osteoprotegerin (OPG) and osteocalcin (OC) are early and late markers of osteoblast differentiation, respectively. To test the expression of these osteogenic markers in YK11-treated cells, their mRNA expression were measured by RT-qPCR. OPG
mRNA expression was increased by YK11 similar to by DHT at day 4 (Fig. 3A) and day 14 (Fig. 3B). OC mRNA expression was increased by YK11 similar to by DHT at day 14 (Fig. 3D), but not at day 4 (Fig. 3C). Furthermore, YK11 increased OC mRNA expression in a dose dependent manner at day 14 (Fig. 3E).

Moreover, induction of OC mRNA expression by YK11 and DHT was prevented in the presence of HF (Fig. 3F). These results also indicate that YK11 promoted osteoblast differen-
tiation in MC3T3-E1 cells similar to DHT.

**Effect of YK11 on Non-genomic Signaling** AR was reported to directly activate the phosphoinositide 3-kinase (PI3K)/Akt pathway, which is a key regulator of osteoblast proliferation and differentiation.25) In this study, we demonstrated that YK11 promotes osteoblast cell proliferation and differentiation via AR (Figs. 1–3). To define the molecular mechanism underlying YK11 activity, we investigated whether YK11 activated Akt signaling in a manner similar to DHT. Cells were treated with YK11 or DHT for 15 min and the levels of phosphorylated Akt protein were investigated. Similar to DHT, phosphorylation of Akt protein increased upon YK11-treatment. This observation suggests that YK11 can activate the Akt signaling pathway via rapid non-genomic signaling similar to DHT (Fig. 4).

**DISCUSSION**

YK11 is a novel steroidal compound that we synthesized previously.24) We demonstrated that YK11 act as a partial agonist of AR in a reporter gene assay.24) However, YK11 shows interesting gene selective transcriptional activation of endogenous target genes. In human AR-positive MDA-MB 453 breast cancer cells, YK11 treatment induced FKBPSI mRNA expression similar to DHT treatment, though YK11 did not induce SARG mRNA expression, which DHT treatment can do.24) Further, we showed that YK11 induced myogenic differentiation similar to DHT. Selective induction of follistatin mRNA was found to be the unique mechanism for YK11 activity, because the induction was not shown by DHT.23) Thus, YK11 is a remarkable novel type of SARM. In this study, we assessed the effect of YK11 on osteogenic activity using MC3T3-E1 cells.

Osteoblast cell proliferation is the first step of bone formation.27,28) In Fig. 1, we showed that YK11 accelerates osteoblast cell proliferation similar to DHT. A previous report has shown that androgens induce Akt-phosphorylation through AR, resulting in osteoblast cell proliferation.29,30) In Fig. 4, we demonstrated that YK11 rapidly increases phosphorylated Akt protein in a 15-min treatment, which suggests that YK11 activates the non-genomic action of AR similar to DHT. Thus, these observations suggest that YK11 accelerates osteoblast cell proliferation by AR-mediated non-genomic activity. AR directly interacts with the p85α regulatory subunit of PI3K in the cytosol.29,30) Interaction between p85α and AR promotes PI3K activation and generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to induce Akt phosphorylation. This interaction was induced by treatment with the AR agonist R1881. Direct interaction is mediated by binding of the N-terminal domain of AR to the SH2 domain of the p85 regulatory subunit. Previously, we showed that YK11 could not induce the NH2- and carboxyl-terminal (N/C) interaction of AR in contrast to DHT. Non-genomic action of AR may not require N/C interaction as YK11 could induce Akt phosphorylation similar to DHT. This observation is important for determining the action of SARMs and further investigation is required.

OPG is an early differentiation marker that is one of the most frequently used markers to demonstrate osteoblast differentiation. OPG is reported to belong to the tumor necrosis factor (TNF) receptor superfamily as a soluble factor that inhibits osteoclast differentiation.31) OPG is a soluble decoy receptor for receptor activator of nuclear factor kappa-B ligand, which activates osteoclasts.31,32) OC expression in osteoblast is used as a valid marker of osteoblast differentiation and mineral deposition because OC is expressed in osteoblasts at the matrix mineralization phase. In Fig. 3, induction of these osteoblast differentiation markers was observed in YK11-treated cells.

In conclusion, we demonstrated that YK11 promoted osteoblast proliferation and differentiation, and increased ALP activity (a marker of osteoblastic maturation) and calcium deposits as stained with alizarin red S (a marker of mineralization) in Fig. 2. Similar to DHT, this activity may be mediated by the non-genomic action of AR. Thus, YK11 is useful as a potential anabolic SARM for androgen deficiency related diseases in men, such as osteoporosis. It is known that the selectivity of SARMs is different of each.33) Thus, to investigate for activity of each SARMs is essential. Since YK11 shows unique myogenic differentiation mechanism in C2C12 cells different from DHT25 and reported SARMs (unpublished observation), YK11 is a novel type of SARMs. This is the first report for the effect of YK11 on different tissue. However, this compound needs to be subjected to further investigations, such as in vivo experiments.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


