Polyamine pathway is associated with muscle anabolic effects by androgen receptor ligand

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Abstract

Background  Muscle wasting is a common condition concomitant with aging. Androgens significantly increase skeletal muscle mass, but the role of the androgen receptor (AR) in skeletal muscle is not well established. TEI-SARM2, a novel selective androgen receptor modulator (SARM), was developed as a pharmaceutical candidate for the treatment of muscle wasting diseases.

Methods  The efficacy and specificity of TEI-SARM2 were analysed in vitro assays, and efficacy was also evaluated in vivo using orchiectomized male rats, rat tail-suspension, rat and mice cancer-induced muscle atrophy models, and female cynomolgus monkeys. Male myofibre-specific AR-knockout (mARKO) mice were orchiectomized to investigate the role of AR in muscle using TEI-SARM2. RNA-seq analysis of bulbocavernosus muscle was performed. The effects of spermidine were evaluated in C2C12 myoblasts.

Results  Selective, potent anabolic effects of TEI-SARM2 in muscle were detected in rats and monkeys. TEI-SARM2 inhibited muscle loss and promoted muscle recovery in a model of muscle disuse and prevented muscle wasting and improved survival rate in cancer models. In vivo and RNA-seq analyses revealed that AR regulates skeletal muscle mass in myofibres and extramyofibres, both directly and indirectly. Among them, polyamine synthesis-related genes were focused, and spermidine treatment could induce C2C12 cell proliferation.

Conclusions  TEI-SARM2 exhibited potent and selective anabolic effects in muscles, as well as tissue specificity in various animal models. The results support that TEI-SARM2 is a promising drug candidate for muscle wasting diseases. TEI-SARM2 induced anabolic effects on skeletal muscle via AR in myofibres as well as other androgen target cells.

Keywords  Androgen receptor; Sarcopenia; Cancer cachexia; SARM; Polyamine

Introduction

Decreased muscle mass and strength result in reduced mobility and quality of life. Higher rates of nursing care and mortality increase health care costs significantly. Therefore, therapies for muscle disorders, such as muscle atrophy, cachexia, sarcopenia, and dystrophy, have been developed using various approaches. Disuse muscle atrophy after hip fracture is a serious injury. Less than half of the people, aged 65 and older, who survive a hip fracture can walk again without any assistance and, in many cases, never regain their former degree of mobility.1,2
The duration of disability is longer than that required for fracture healing, implying that the hip fracture may trigger other adverse consequences that result in disability. Especially, reduced mechanical loading after hip fracture can induce muscle atrophy. Monoclonal antibody that binds to type II activin receptors (ActRII), which inhibit the binding of myostatin and the receptor, and selective androgen receptor modulators (SARMs) were developed for the treatment of recovery for the patients with hip fracture. However, there is still no commercially available drug for this purpose.

Cachexia is a devastating multifactorial syndrome characterized by severe and involuntary loss of muscle mass and metabolic abnormalities. Cachexia is not fully reversible using conventional nutritional support and eventually leads to functional impairment. Cancer cachexia accounts for nearly 30% of cancer-related deaths. However, few therapeutic options are currently available for cancer cachexia. Some clinical trials had failed to prove the effectiveness of ostarine (GTx, Inc., non-steroidal SARM) and anamorelin, which mimics ghrelin, an appetite-stimulating peptide hormone mainly produced by the stomach. There is a lot of debate about why the trials failed to show functional improvements; however, it can be considered that those drug candidates might have not good enough anabolic effects to recover the cachexia.

It is well known that androgens can increase muscle mass and strength, but they are not routinely used to treat these disorders because of their unfavourable side effects in cardiovascular, liver, and reproductive tissues. The androgen receptor (AR) is a nuclear receptor that plays a critical role in the function of several organs including the prostate, testis, ovary, skeletal muscle, and bone. AR is activated by binding to ligands such as androgens, after which it translocates into the nucleus, where it binds to androgen response elements within the promoters or enhancers of androgen-responsive genes such as E-cadherin and fibroblast growth factor 8. Co-factors are then recruited to the AR dimer complex, which is thereby activating gene transcription. AR has a C-terminal ligand-binding domain, which encompasses the activation function 2 (AF2) domain. This AF2 domain of AR was shown to mediate the agonist-induced interaction between the NH2- and COOH-terminal (N/C) domains of AR, and this interaction in turn regulates the function of AR in response to agonists. The AR gene is widely expressed in myoblasts, myofibres, satellite cells, and motor neurons, which are direct targets of androgens and may contribute to the regulation of muscle mass or strength. However, the role of AR in skeletal muscle is not well established.

Steroidal androgens, such as nandrolone, oxandrolone, and fluoxymesterone, have beneficial effects on muscle and bone but are not widely used due to safety concerns. SARMs are tissue-selective AR ligands that have demonstrated superior safety profiles to those of androgenic anabolic steroids in preclinical and clinical studies. SARMs have been shown to exhibit anabolic activity in muscles but few effects in reproductive tissues. TEI-SARM2 was developed at Teijin Pharma Ltd. as a selective non-steroidal SARM. TEI-SARM2 was selected by screening tests for the ability to AR binding affinity and AR-dependent transcriptional activity from large number of compounds. In addition, it was necessary for us to discover the candidate compound that has the strong anabolic effects for skeletal muscle with minimal effects in reproductive tissues. TEI-SARM2 has a long half-life and high area under the blood concentration–time curve (AUC) in blood, enabling a once-weekly treatment regimen. TEI-SARM2 has a longer half-life and higher AUC in blood than those of ostarine, the clinical effects of which have been evaluated most extensively among the current SARMs.

Based on this background, it has been desired to be developed that novel therapeutic strategies including AR agonists such as TEI-SARM2 to treat skeletal muscle atrophy or wasting due to several conditions such as disuse or cancer cachexia. However, the anabolic actions of androgens in skeletal muscle remain elusive. In this study, we evaluated the potential efficacy of TEI-SARM2 in various types of skeletal muscle atrophies and investigated the role of AR in skeletal muscle using myofibre-specific AR-knockout (mARKO) mice treated with TEI-SARM2.

Materials and methods

Androgen receptor binding assay

The binding affinity of the compounds, including dihydrotestosterone (DHT), ostarine, nandrolone, and TEI-SARM2, to AR was determined via a competitive ligand binding assay. The AR ligand binding assay was performed using the PolarScreen Androgen Receptor Competitor Assay, Red (Invitrogen). The AR ligand-binding domain was tagged with glutathione-S-transferase using the PolarScreen Androgen Receptor Competitor Assay, Red (Invitrogen).

Transactivation assay

HEK293 cells were grown in 96-well plates and transfected with the plasmids indicated below using Lipofectamine 2000 reagent (Invitrogen) for 3 h. After transfection, compounds, including DHT, ostarine, nandrolone, and TEI-SARM2, were added to the cells for 24 h, followed by measurement of luciferase activity using Dual-Glo Luciferase Assay System (Promega). The pGL4-3xARE-Luc, pHRL-TK (Promega), and pcDNA-hAR plasmids were used to evaluate AR transactivation in ARE reporter assay, the pM-hAR_AF2, pVP16-hAR_AF1, pGL4-GAL4x5-Luc, and pHRL-TK plasmids to evaluate N/C interaction in mammalian two-hybrid assay, and the pM-hAR_AF2, pGL4-GAL4x5-Luc, and pHRL-TK plasmids to evaluate AF2 activation in mammalian one-hybrid assay. Data were fit to a four-parameter-fit
logistics to determine EC\textsubscript{50} values. The Emax value was calculated as a percentage of that obtained by \(10^{-7} \) M DHT treatment.

**Animals and interventions**

The animal experiments were performed at four institutions. All procedures were approved by the institutional animal care and use committees of Teijin Ltd. (TEI-SARM2 daily and weekly dosing and selectivity experiments in male Sprague–Dawley rats and disuse muscle atrophy experiments in female Wistar rats), KAC Co., Ltd. (MKN45 tumour-bearing mice and AH-130 tumour-bearing rats experiments), Shin Nippon Biomedical Laboratories, Ltd. (cynomolgus monkey experiment), and Ehime University (skeletal mARKO mouse experiments).

**Tissue selectivity of TEI-SARM2 in Sprague–Dawley rats**

Male Sprague–Dawley rats, 8 weeks old (Charles River Laboratories Japan, Inc.), were maintained under a regular dark/light cycle (light from 6:00 to 18:00) with free access to food and water during the entire experiment, including the night before euthanasia. After an acclimation period, Sprague–Dawley rats were randomized according to body weight. Rats were treated with TEI-SARM2 (0.03–3 mg/kg, weekly, p.o.) or nandrolone decanoate (ND) (0.3–3 mg/kg, weekly, s.c.) for 6 weeks. The levator ani (LA) muscle and prostate were weighed after euthanasia.

**Anabolic effects of TEI-SARM2 daily/weekly dosing in Sprague–Dawley rats**

Male Sprague–Dawley rats, 8 weeks old, were purchased from Charles River Laboratories Japan, Inc. and maintained under a regular dark/light cycle (light from 6:00 to 18:00) with free access to food and water during the entire experiment, including the night before euthanasia. After an acclimation period, the rats were randomized according to body weight and orchiectomized (ORX) under anaesthesia. On Day 14 after ORX, the rats were treated with TEI-SARM2 (0.03–1 mg/kg, daily, or 1–10 mg/kg, weekly, p.o.) or nandrolone (ND; 1–90 mg/kg, one-time administration, s.c.) for another 14 days. On Day 28, the mice were euthanized, and the LA muscle was weighed.

**Disuse muscle atrophy model**

Female Wistar rats, 10 weeks old, were purchased Japan SLC, Inc. The rats were maintained under a regular dark/light cycle (light from 6:00 to 18:00) with free access to food and water during the entire tail-suspension experiment including the night before euthanasia. After an acclimation period, the rats were assigned to nine groups (three intact groups: \(n = 4\); six tail-suspension groups: \(n = 9\)). In the tail-suspension groups, rats were suspended by the tail to avoid contact between the hindlimb and ground. Vehicle or 30 mg/kg TEI-SARM2 was administered orally once a week for 28 days from the first day of suspension. On Day 14, the rats were released from suspension and allowed to recover for another 14 days. The weight of the gastrocnemius muscle was measured on Days 14, 21, and 28 after euthanasia.

**Cancer cachexia models**

Female BALB/c Slc-nu/nu mice, 5 weeks old, were purchased from Japan SLC, Inc. and maintained under a regular dark/light cycle (light from 7:00 to 19:00) with free access to water. MKN45 human gastric cancer cells (3 \(\times 10^7\)/mouse) were inoculated subcutaneously into 6-week-old mice. MKN45 cell-implanted mice were treated with TEI-SARM2 (1 or 10 mg/kg, daily, p.o.), ND (10 mg/kg, biweekly, s.c.), or vehicle for 4 weeks. We monitored the volume of food consumption during the study period. Body, tumour, gastrocnemius muscle, white adipose tissue, and heart weights were measured after euthanasia.

Female Wistar rats, 7 weeks old, were purchased from Japan SLC, Inc. and maintained under a regular dark/light cycle (light from 7:00 to 19:00) with free access to water. Yoshida AH-130 hepatoma cells (1 \(\times 10^6\)/mouse) were inoculated intraperitoneally into 8-week-old rats (Day 0). Body weight and food consumption were monitored for 3 weeks. AH-130 cell-implanted rats were treated with TEI-SARM2 (1 or 10 mg/kg, daily, p.o.) or vehicle for 3 weeks.
samples for aspartate transaminase, alanine transaminase, alkaline phosphatase, γ-glutamyltransferase, creatine kinase, and total bilirubin were performed at Day 27.

**Generation of mARKO mice**

The HSA-Cre$^{22}$ and AR$^{l−2/l+23}$ mouse strains have been described previously. Female AR$^{l−2/l}$ mice were crossed with male HSA-Cre mice to generate male HSA-Cre:AR$^{l−2/Y}$ mARKO mice. All mice were housed in a specific pathogen-free facility under climate-controlled conditions and a 12 h light/dark cycle and were provided water and a standard diet *ad libitum*. All animals were 7–12 weeks of age at the time of evaluation.

**Histological analysis of skeletal muscle**

Muscles were frozen in liquid nitrogen-chilled isopentane, and 10 μm cryosections were collected for immunofluorescence staining. For AR immunostaining, sections were incubated with an AR antibody (Keyence) overnight at 4°C. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) was used as the secondary antibody. Stained tissues were photographed using BZ-9000 BioRevo (Keyence).

**ORX male mARKO mice treated with androgens**

Male mARKO and littermate control (AR$^{l−2/Y}$) mice underwent ORX or not. Nine days after ORX, the mice were treated with TEI-SARM2 (10 mg/kg, weekly, p.o.), ND (100 mg/kg, bi-weekly, s.c.), or vehicle (weekly, p.o.) for another 2 weeks. The forelimb strength of the mice was measured by the grip test on Day 21. After euthanasia, the LA and bulbocavernosus (BC) muscles were weighed. RNA-seq and quantitative reverse-transcription PCR (qRT-PCR) were performed using RNA extracted from BC muscles.

**Grip strength test in littermate control and mARKO mice after ORX**

A blinded test of forelimb grip strength was performed using a commercial digital grip strength metre (GPM-100B, Melquest). Mice held by the tail were gently allowed to grasp a wire grip with their forepaws. The mice were then gently pulled by the tail until they released their grip. The force achieved by the mouse was averaged over five trials.

**Quantitative reverse-transcription PCR**

Total RNA was extracted using TRizol reagent (Life Technologies) and the RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). qRT-PCR was performed on the 7500 Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). The oligonucleotide primers used were as follows: mouse ornithine decarboxylase 1 (Odc1), 5′-GGGTTGACATCATGGAAG-3′ and 5′-TGTGTCGTAG-3′; mouse insulin-like growth factor 1 (Igf1), 5′-GTATGCTGCTCCAGCTG-3′ and 5′-TCGGAGAGCAACATCTACCTG-3′; mouse myogenin, 5′-CTACAGGCCCTTGCTCAGTCT-3′ and 5′-AGATTGTGCGCTCTGTAAGG-3′; mouse MyoD, 5′-AGCACATACGTGGGACTCA-3′ and 5′-GCCTCACTATGCT-3′; mouse Ptgds, 5′-GGGGCAAAAGACCTGGA-3′ and 5′-AGGTCTCTCACGGACTGTAAG-3′; mouse Car6, 5′-GCCGCTGTCGCTGCTT-3′ and 5′-CTCTCCACGGCCTC-3′; mouse Ptgds, 5′-GGTTCCTGACACATACCTA-3′ and 5′-ATAGTTGGCCTCCACCACTG-3′; mouse Ct, 5′-TCGCCAAGTTGCGCAAGAG-3′ and 5′-AGTTCTCTCCAAGGCAGTA-3′; mouse Mup4, 5′-AACGCGCCAGAATGGAGAA-3′ and 5′-CAATGTGCTTGCTTCAGGG-3′; mouse Hprt, 5′-GGCTGTGTGTCGCTGCTTCTC-3′ and 5′-TTCCACGGCGCACTC-3′; mouse Hprt, 5′-GGCTGTGTGTCGCTGCTTCTC-3′ and 5′-TTCCACGGCGCACTC-3′; mouse Hprt, 5′-GGCTGTGTGTCGCTGCTTCTC-3′ and 5′-TTCCACGGCGCACTC-3′; mouse Hprt, 5′-GGCTGTGTGTCGCTGCTTCTC-3′ and 5′-TTCCACGGCGCACTC-3′; mouse Hprt, 5′-GGCTGTGTGTCGCTGCTTCTC-3′ and 5′-TTCCACGGCGCACTC-3′.

Other sequences of interest: 5′-GGGTATCTGTG-3′ and 5′-CTTTTAATGTGAC-3′ and 5′-GGGGTATCTGTG-3′ and 5′-CTTTTAATGTGAC-3′.

**RNA-seq**

RNA-seq was performed with three replicates in each group. The integrity of isolated RNA was verified using the Agilent 2100 Bioanalyzer. RNA samples with an RNA integrity number >8 were normalized to 100 ng/μL before further analyses. RNA-seq libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Prep Kit setA, according to the manufacturer’s instructions, and subsequently confirmed to comprise fragments with an average size of approximately 330–340 bp using the 2100 Bioanalyzer and Agilent DNA1000 kit. Sequencing of paired-end reads (75 bp) was performed using the MiSeq Reagent Kit v3, 150 cycles, on the MiSeq platform (Illumina). Sequencing data were mapped to the mouse genome (mm10) using TopHat2 and were analysed using the Mouse Genome Annotation Project (mm10).
Anabolic effects by AR ligand is related with polyamine signaling

Cufflinks. Data were registered at NCBI Gene Expression Omnibus (GEO) with accession number GSE153147.

Analysis of sequencing data

According to the RNA-seq data, differentially expressed genes (DEGs) considered with the cut-off at false discovery rate < 0.05, defined as those with expression levels that were significantly increased or decreased in mARKO mice by more than twofold that of the littermate control, were extracted for further analyses. A heatmap was generated using MeV, and gene ontology analyses were performed using DAVID Bioinformatic Resources 6.7 and gene set enrichment analysis.

Cell culture

C2C12 mouse myoblasts (CRL-1722, ATCC) were seeded in a 96-well plate at 2 × 10^3 cells per well and cultured in Dulbecco’s modified Eagle’s medium (Gibco), supplemented with 4 mM glutamine and 10% foetal bovine serum for 24 h. To determine the influence of a polyamine on myoblasts, C2C12 cells were incubated with spermidine (Sigma-Aldrich) at a final concentration of 6–2000 nM for 48 h. Subsequently, cell proliferation was measured using CellTiter-Glo (Promega).

Statistical analysis

All data are expressed as means ± standard deviation. For two-group comparisons, Student’s t-test was used. For multiple comparisons, one-way analysis of variance followed by Dunnett’s t-test was used to compare each group with the vehicle-treated group. For comparisons among multiple time points, two-way analysis of variance was performed for between-group comparisons, followed by the Bonferroni test for comparisons at specific time points. All statistical analyses were performed using GraphPad Prism software, version 7 (GraphPad). Differences with P < 0.05 were considered significant.

Results

Binding and transcriptional activities of androgen receptor

The AR binding activity (50% inhibitory concentration, IC_{50}), transcriptional activity (50% maximal concentration, EC_{50}), N/C interaction, and AF2 activity induced by DHT, TEI-SARM2, ND, and ostarine are shown in Table 1. TEI-SARM2 is a pure AR agonist without AR antagonist activity, whereas ostarine possesses AR antagonist activity. TEI-SARM2 induced potent AR binding and transcriptional activities comparable with those induced by ostarine, the most well-investigated SARM in clinical studies. Interestingly, TEI-SARM2 induced AR N/C interaction; these effects were comparable with those of DHT and ND, a clinically available anabolic steroid, whereas ostarine induced minimal effects. These data support TEI-SARM2 as a pharmaceutical candidate for the treatment of muscle wasting diseases.

Differentiation between the anabolic and androgenic effects of TEI-SARM2

An in vivo dose–response study in normal rats was performed to differentiate the anabolic from androgenic effects of TEI-SARM2 using both anabolic (LA muscle) and androgenic (prostate) tissues. TEI-SARM2 (p.o.) at 3 mg/kg weekly increased LA muscle weight in a dose-dependent manner up to 140% of the weight in the control group (vehicle treatment) (Figure 1A), whereas no such change was observed in the prostate (Figure 1B). ND (s.c.) at 3 mg/kg weekly did not significantly change LA muscle weight but decreased prostate weight to 29% of the control group weight (Figure 1). In contrast, testis weight was not affected by the treatments (Figure 1C). These results indicate that the anabolic effects of TEI-SARM2 were tissue selective, at least in the case of LA muscle vs. prostate tissues.

Table 1 In vitro profiles of androgen receptor agonists

|          | AR binding (IC_{50}, nM) | AR transcriptional activity (EC_{50}, nM) | N/C interaction | AF2 activity |
|----------|--------------------------|-----------------------------------------|-----------------|-------------|
|          | EC_{50} (nM) | Emax (%) | EC_{50} (nM) | Emax (%) |
| DHT      | 10             | 0.61     | 0.19          | —           | 0.36        | —           |
| TEI-SARM2| 27             | 37       | 186           | 95          | —           | 9           |
| Ostarine | 13             | 15       | —             | 30          | —           | 0           |
| Nandrolone| 26             | 0.46     | 0.82          | 70          | 1.3         | 70          |

AR binding: fluorescent-labelled ligand competitive assay. AR transcriptional activity: luciferase assay in HEK293 cells. N/C interaction: mammalian two-hybrid assay in HEK293 cells. Emax is shown as % compared with that of 10^{-7} M DHT. AF2 activity: mammalian one-hybrid assay in HEK293 cells. Emax is shown as % compared with that of 10^{-7} M DHT.
**Figure 1** TEI-SARM2 selectivity for muscle rather than the prostate and testis in normal male rats, and effect of TEI-SARM2 in orchiectomized male rats. Male Sprague–Dawley rats (9 weeks old) were treated with TEI-SARM2 (0.3, 1, and 3 mg/kg, weekly, p.o.) or ND (0.3, 1, and 3 mg/kg, weekly, s.c.) for 6 weeks. The LA muscle (A), prostate (B), and testis (C) were weighed after euthanasia and presented as a percentage of body weight (BW). Data are presented as means ± standard deviation (SD) (n = 5 per group). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. (D) Male Sprague–Dawley rats (9 weeks old) were orchiectomized. On Day 14 after orchiectomy, rats were treated with TEI-SARM2 (0.01–1 mg/kg, daily, p.o.) or nandrolone (ND: 1–100 mg/kg, one-time administration, s.c.) for another 14 days. The levator ani (LA) muscle was weighed after euthanasia on Day 28. Data are presented as means ± SD (n = 5 per group). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with sham treatment, by Student’s t-test. (E) Male Sprague–Dawley rats (9 weeks old) were orchiectomized. On Day 14 after orchiectomy, rats were treated with TEI-SARM2 (1–10 mg/kg, weekly, p.o.) or ND (10–90 mg/kg, one-time administration, s.c.) for another 14 days. The LA muscle was weighed after euthanasia on Day 28. Data are presented as means ± SD (n = 5 per group). ** and *** indicate P < 0.01 and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with sham treatment, by Student’s t-test.

**Anabolic effects of TEI-SARM2**

Muscle loss was induced in male rats by in vivo castration, and the anabolic effects of TEI-SARM2, represented by recovery of LA muscle weight, were investigated. In this model, animals received oral TEI-SARM2 daily for 14 days starting on Day 14 after castration. The LA muscle was weighed on Day 28 and calculated as a percentage of body weight. LA muscle weight in ORX rats was 54% of that in the sham group. TEI-SARM2 increased LA muscle weight in a dose-dependent manner from 0.03 to 1 mg/kg. TEI-SARM2 treatment at 0.1 mg/kg daily for 14 days induced recovery of LA muscle weight almost to the weight in the sham group (**Figure 1D**). ND (s.c. administration on Day 14) also induced anabolic effects in a dose-dependent manner (**Figure 1D**). Weekly dosing was conducted using the same experimental procedure as for daily dosing. LA muscle weight in ORX rats was 40% of that in the sham group. TEI-SARM2 increased LA muscle weight in a dose-dependent manner from 1 to 30 mg/kg. At 1 mg/kg weekly for 14 days, TEI-SARM2 induced recovery of LA muscle weight almost to the weight in the sham group (**Figure 1E**). The Emax values for muscle weight were comparable between the TEI-SARM2 and ND treatments (**Figure 1**). These data showed that TEI-SARM2 exhibits potent anabolic effects.
comparable with those of other anabolic steroids, regardless of a daily or weekly dosing schedule.

**Efficacy of TEI-SARM2 on disuse muscle atrophy**

The effect of TEI-SARM2 on disuse muscle atrophy was evaluated in a rat tail-suspension test (Figure 2A). The acute and recovery phases may reflect the condition of bedrest after surgery for hip fracture and rehabilitation from the surgery, respectively. Weekly oral dosing of TEI-SARM2 significantly prevented rapid loss of muscle weight during tail-suspension as the acute phase. Further, TEI-SARM2 increased the weight of the gastrocnemius muscle (Figure 2B), but not the soleus (Figure 2C), to the level in intact control animals within 2 weeks after reloading hindlimb in recovery phase. These results indicate that TEI-SARM2 prevents muscle atrophy and promotes muscle recovery under atrophic conditions.

**Efficacy of TEI-SARM2 on cancer cachexia and mortality**

MKN45 gastric cancer cell xenograft mice were used to assess the efficacy of TEI-SARM2. As a result of cachexia induced by the transplanted gastric cancer cells, mice lost 7.2% of their body weight at 28 days after transplantation, compared with a gain of 25.5% body weight in mice in the intact control group. TEI-SARM2 at 10 mg/kg daily for 4 weeks significantly suppressed this weight loss, increasing body weight to 109% compared with the vehicle-treated group (Figure 3A), without affecting food intake (Figure 3B) or gastric cancer cell growth (Figure 3C). TEI-SARM2 also prevented reductions in muscle weight (Figure 3D), white adipose tissue (Figure 3E), and heart weight (Figure 3F). The ND dose (10 mg/kg) appeared sub-optimum in this experiment, as well. In addition, TEI-SARM2 treatment could not affect the expression levels of FoxO1, FoxO3, and atrogens, such as Atrogen-1 and MuRF1; however, the expression levels of reported androgen/AR target genes in skeletal muscle, such as Igf1 and Odc1, were increased (Supporting Information, Figure S1). These results showed that TEI-SARM2 leads to recovery of the body and muscle weights reduced by cachexia, without affecting expression of atrogens in skeletal muscle and any effect on tumour tissues.

To assess the effect of TEI-SARM2 on cancer-associated mortality, the survival rate of rats with cancer cachexia induced by AH-130 hepatoma cell allografts was evaluated. Cachexia in these rats resulted in suppression of body weight gain (Figure 3G) and food intake (Figure 3H) from Days 0 to 10 in the vehicle group compared with the intact control group. Daily dosing of TEI-SARM2 at 1 and 10 mg/kg significantly increased body weight (Figure 3G), with no change in food intake, compared with the vehicle group on Day 10. On Day 21 (Figure 3H), the overall survival rates of rats treated with vehicle, 1 mg/kg TEI-SARM2, and 10 mg/kg TEI-SARM2 were 45%, 75%, and 70%, respectively (Figure 3I). Treatment with 1 and 10 mg/kg TEI-SARM2 significantly reduced mortality in AH-130 cell allograft rats. These findings indicate that the potent anabolic effect of TEI-SARM2 improves survival in rats with cancer cell-induced cachexia.

*Figure 2* Effect of TEI-SARM2 on disuse muscle atrophy. Female Wistar rats (12 weeks old, n = 4 or 9) were suspended by the tail to avoid contact between the hindlimb and ground. On Day 14, the rats were released from suspension and allowed to recover for 14 days. Muscle weight was measured on Days 14, 21, and 28. From the first day of suspension, 30 mg/kg TEI-SARM2 was administered orally once weekly for 28 days. Data are presented as means ± standard deviation (intact control groups: n = 4; tail-suspension groups: n = 9). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, by Tukey’s test.
Figure 3  Effect of TEI-SARM2 on female mouse and rat model of cachexia. BALB/c nu/nu female mice (6 weeks old) were transplanted with MKN45 human stomach adenocarcinoma cells and then administered oral TEI-SARM2 at 1 or 10 mg/kg daily for 4 weeks. ND at 10 mg/kg was administrated once-biweekly subcutaneously for 4 weeks. Body weight (A), food consumption (B), tumour weight (C), gastrocnemius muscle (D), white adipose tissue (WAT, E), and heart (F) were weighed after euthanasia. Data are presented as means ± standard deviation (n = 6 per group). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with sham treatment, by Student’s t-test. Female Wistar rats (8 weeks old) were inoculated intraperitoneally with Yoshida AH-130 hepatoma cells and orally administered TEI-SARM2 at 1 or 10 mg/kg daily for a maximum of 21 days. Body weight (G), food consumption (H), and survival rate (I) were recorded for 21 days. Data are presented as means ± standard deviation (n = 10, 40, 20, and 20 in the control, vehicle, 1 mg/kg TEI-SARM2, and 10 mg/kg TEI-SARM2 groups, respectively). *** indicates P < 0.001, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with intact mice, by Student’s t-test. a and b indicate P < 0.05 and P < 0.01, respectively, by generalized Wilcoxon’s test.
Anabolic effect of TEI-SARM2 in female cynomolgus monkeys

The effects of TEI-SARM2 were also examined in non-human primates, in addition to rodents. Biochemical analyses showed that treatment with more than 1 mg/kg of TEI-SARM2 significantly increased alanine transaminase and γ-glutamyltransferase levels (Table 2). TEI-SARM2 administered at 0.1 mg/kg for 4 weeks significantly increased body weight by 3.1 kg, compared with 2.5 kg in the vehicle group.

Table 2  Blood chemistry profile of TEI-SARM2 in female cynomolgus monkeys

| Parameters | Control | 0.1 | 1 | 10 |
|------------|---------|-----|---|----|
| Serum | | | | | |
| AST (IU/L) | 55.3 ± 41.7 | 76.2 ± 42.7 | 130.5 ± 88.3 | 127.8 ± 42.0 |
| ALT (IU/L) | 49.0 ± 15.2 | 93.5 ± 59.7 | 187.5 ± 118.6* | 330.5 ± 181.5** |
| ALP (IU/L) | 1294.5 ± 816.7 | 1451.8 ± 462.4 | 1571.0 ± 665.1 | 1826.5 ± 766.9 |
| GGT (IU/L) | 62.2 ± 17.1 | 206.2 ± 107.0 | 415.8 ± 214.7* | 534.0 ± 199.7** |
| CK (IU/L) | 562.2 ± 718.7 | 768.3 ± 601.3 | 1191.2 ± 1830.8 | 558.7 ± 414.5 |
| T-Bil (mg/dL) | 0.137 ± 0.043 | 0.115 ± 0.029 | 0.128 ± 0.043 | 0.188 ± 0.043 |

Results are mean ± standard deviation. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CK, creatine kinase; GGT, γ-glutamyltransferase; T-Bil, total bilirubin.

Mean ± standard deviation, n = 6, Dunnett’s test, vs. control.

*P < 0.05.

**P < 0.01.

***P < 0.001.

Figure 4  Effect of TEI-SARM2 in female cynomolgus monkeys. Female cynomolgus monkeys (3–4 years old) were treated with TEI-SARM2 at 0.1, 1, or 10 mg/kg daily for 4 weeks. Body weight was recorded for 24 days. Lean body mass (LBM) was measured on Day 24. Baseline indicates the LBM before dosing during the acclimation period. Body weight (A), LBM of forelimb (B), LBM of hindlimb (C), and LBM of trunk (D). Data are presented as means ± standard deviation (n = 6 per group). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test.
on Day 27. This increase was also observed at TEI-SARM2 doses of 1 and 10 mg/kg (increases of 3.1 and 3.3 kg, respectively). All three doses resulted in gradual weight gain in the monkeys from Days 0 to 27 (Figure 4A).

The LBMs of the forelimb (Figure 4B), hindlimb (Figure 4C), and trunk (Figure 4D) were measured on Day 24. All LBMs were significantly increased by TEI-SARM2 in a dose-dependent manner up to 10 mg/kg (Figure 4). These data indicate no difference in the potent anabolic effects of TEI-SARM2 between rodents and non-human primates.

**The role of androgen receptor in skeletal muscle using TEI-SARM2-treated mARKO mice**

Androgen receptor expression in skeletal muscle was successfully decreased in mARKO mice compared with littermate control mice (Figure 5A). Male mARKO and littermate control mice underwent ORX or not. Nine days after ORX, mice were treated with TEI-SARM2 (10 mg/kg, weekly, p.o.), ND (100 mg/kg, biweekly, s.c.), or vehicle (weekly, p.o.) for another 2 weeks. In non-ORX mice, the LA and BC muscle

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**Figure 5** The role of AR in skeletal muscle using TEI-SARM2-treated HSA-Cre;AR<sup>L2Y</sup> (mARKO) mice. (A) Expression of AR in the hindlimb muscle of mARKO mice. Transverse sections of tibialis anterior (TA), gastrocnemius (Ga), and quadriceps (Qu) muscles. Blue: DAPI, yellow: AR, white: AR positive nuclei. Scale bar = 50 μm. (B–D) Effect of TEI-SARM2 on LA/BC muscle, prostate weights, and grip strength in control and mARKO mice. mARKO mice and wild-type (WT) littermates (14 weeks old) were orchiectomized. On Day 9 after orchiectomy, mice were treated with TEI-SARM2 (10 mg/kg, weekly, p.o.) or ND (100 mg/kg, one-time administration, s.c.) for another 14 days. The levator ani (LA) and bulbocavernosus (BC) muscles (B) and prostate (C) were weighed after euthanasia on Day 23 and are expressed as a percentage of body weight (BW). Forelimb strength (D) was measured by the grip test on Day 21. Data are presented as means ± standard deviation (n = 4 per WT group, and n = 5, 6, 7, and 6 in the sham, vehicle, TEI-SARM2, and ND treatment groups of mARKO mice, respectively). * and *** indicate P < 0.05 and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with sham treatment, by Student’s t-test.
weights were significantly lower in mARKO mice than in control mice, and a further reduction in muscle weight was found in mARKO mice after ORX (Figure 5B). In addition, the TEI-SARM2 and ND treatments ameliorated the ORX-induced LA and BC muscle losses in both mARKO and control mice (Figure 5B). Prostate weight was unchanged between mARKO and control mice, regardless of receiving ORX by either TEI-SARM2 or ND treatment (Figure 5C). Gastrocnemius and tibialis anterior muscle weights were slightly but significantly lower in mARKO mice than in control mice, although muscle weight of hindlimb except TA was not affected by receiving ORX, either TEI-SARM2 or ND treatment (Table 3). On the other hand, TEI-SARM2 and ND treatments ameliorated the reduction of grip strength induced by ORX in mARKO mice (Figure 5D). In addition, qRT-PCR analysis in BC muscle revealed that the expression of known target genes of AR in skeletal muscle, including Odc1 and Igf1 (Figure 6), but not well-known AR target genes such as Srd5a1, Sts, and Ncoa1 (Figure S2), was up-regulated by TEI-SARM2 regardless of mARKO mice. In addition, autophagy-related genes were up-regulated by TEI-SARM2. LC3b was up-regulated in an AR-independent manner. Lamp1 and Lamp2 were up-regulated by TEI-SARM2 in an AR-dependent manner (Figure 6). These results suggest that TEI-SARM2 exerts its anabolic effects both directly and indirectly via myofibre AR including autophagy and these gene expressions by AR can be tissue specific.

RNA-seq analysis of the BC muscle in ORX and non-ORX mARKO mice treated with TEI-SARM2

The effect of TEI-SARM2 on transcriptional profiles in skeletal muscle was examined by RNA-seq. Up-regulated genes were identified in sham control vs. mARKO mice, ORX control vs. ORX mARKO mice, and ORX control vs. ORX mARKO mice treated with TEI-SARM2. Venn diagram analysis revealed that 20 DEGs were common among these three comparisons (Figure 7A, Table S1). These genes might be considered direct target genes of skeletal myofibre AR. In addition, to identify indirect target genes of myofibre AR, genes up-regulated or down-regulated in the ORX control and mARKO mice treated with TEI-SARM2 compared with the sham control were extracted. Venn diagram analysis revealed that six DEGs (Mup20, Car6, Ptgds, C7, Mup4, and 9130230L2Rik) were common among the four comparisons (Figure 7B, Table S2). In addition, qRT-PCR analysis in BC muscle revealed that the expression of Mup20, Car6, Ptgds, C7, and Mup4 was

| Table 3 Effects of TEI-SARM2 or ND in orchietomized or sham-operated mARKO mice on muscle weight |
|---------------------------------------------------------------|
| Parameters | Control |   |   |
|            | Vehicle (4) | Vehicle (4) | TEI-SARM2 (4) | ND (4) |
| Muscle weight |   |   |   |   |
| LA/BC | 100 ± 6.73 | 53.1 ± 4.42* | 77.9 ± 3.47*** | 88.4 ± 6.71*** |
| GAS | 100 ± 4.81 | 101 ± 2.20 | 92.2 ± 2.37*** | 94.3 ± 0.330** |
| Soleus | 100 ± 8.77 | 110 ± 9.45 | 94.9 ± 2.19* | 103 ± 7.89 |
| EDL | 100 ± 5.62 | 98.9 ± 1.24 | 94.6 ± 1.54 | 99.9 ± 5.70 |
| TA | 100 ± 3.35 | 91.7 ± 5.28| 92.6 ± 4.77 | 99.5 ± 3.81 |
| Heart | 100 ± 4.03 | 96.5 ± 8.48 | 94.2 ± 2.67 | 94.4 ± 7.09 |
| mARKO |   |   |   |   |
| Parameters | Vehicle (5) | Vehicle (6) | TEI-SARM2 (7) | ND (6) |
| Muscle weight |   |   |   |   |
| LA/BC | 49.4 ± 3.63* | 27.6 ± 3.25* | 42.5 ± 3.67*** | 48.1 ± 4.09*** |
| GAS | 91.4 ± 1.54* | 96.9 ± 5.43 | 97.2 ± 3.02 | 96.9 ± 3.58 |
| Soleus | 103 ± 11.7 | 107 ± 3.04 | 109 ± 5.44 | 102 ± 5.10 |
| EDL | 96.6 ± 5.07 | 95.2 ± 6.14 | 96.5 ± 4.79 | 93.4 ± 3.19 |
| TA | 93.6 ± 2.33* | 94.7 ± 5.20 | 95.2 ± 2.71 | 92.0 ± 4.30 |
| Heart | 95.6 ± 6.58 | 86.1 ± 3.09* | 99.5 ± 12.0 | 102 ± 11.8* |

Results are mean ± standard deviation for the number of animals indicated in parentheses. Muscle weight is expressed as a ratio of sham-operated control. LA/BC, levator ani/bulbocavernosus muscle; GAS, gastrocnemius muscle; EDL, extensor digitorum longus; TA, tibialis anterior muscle. Mean ± standard deviation, n = 4–7, t-test, vs. mARKO, vs. sham. *P < 0.05. Dunnett’s test, vs. control. **P < 0.05. ***P < 0.01. ****P < 0.001.
Figure 6 mRNA levels in the BC muscle of mARKO mice. mARKO mice and WT littermates (14 weeks old) were orchiectomized. On Day 9 after orchiectomy, the mice were treated with TEI-SARM2 (10 mg/kg, weekly, p.o.) or ND (100 mg/kg, one-time administration, s.c.) for another 14 days. The levator ani (LA) and bulbocavernosus (BC) muscles were collected after euthanasia on Day 23. Igf1, Odc1, myogenin, MyoD, Map1lc3b, p62, lamp1, and lamp2 mRNA levels in BC muscle were measured by qRT-PCR. Data are presented as means ± standard deviation (n = 4 per WT group, and n = 5, 6, 7, and 6 in the sham, vehicle, TEI-SARM2, and ND groups of mARKO mice, respectively). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with sham treatment, by Student’s t-test.
Figure 7 RNA-seq analysis and mRNA levels in BC muscle of mARKO mice. (A) Venn diagram of DEGs identified by pairwise comparisons of sham WT vs. sham mARKO mice, ORX WT vs. ORX mARKO mice, and ORX WT mice treated with TEI-SARM2 vs. ORX mARKO mice treated with TEI-SARM2. DEGs were identified using a false discovery rate cut-off <0.05 and a fold change cut-off >2. (B) Venn diagram of DEGs identified by pairwise comparisons of sham WT vs. ORX WT, ORX WT treated with TEI-SARM2 vs. ORX WT treated with vehicle, sham mARKO vs. ORX mARKO, and ORX mARKO treated with TEI-SARM2 vs. ORX mARKO treated with vehicle. DEGs were identified using a false discovery rate cut-off <0.05 and a fold change cut-off >2. WT, littermate mice; ORX WT, orchiectomized WT mice treated with vehicle. (C–G) Mup20, Car6, Ptgds, C7, and Mup4 were decreased by ORX and up-regulated by TEI-SARM2 in muscle AR-independent manner. Those mRNA levels in BC muscle were measured by qRT-PCR. Data are presented as means ± standard deviation (n = 4 per WT group, and n = 5, 6, 7, and 6 in the sham, vehicle, TEI-SARM2, and ND groups of mARKO mice, respectively). *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates P < 0.001, compared with sham treatment, by Student’s t-test.
up-regulated by TEI-SARM2 regardless of mARKO mice (Figure 7C–G). These genes might be considered indirect target genes of AR in extra-myofibre tissues or cells.

**Treatment of mouse C2C12 myoblasts with a polyamine**

Odc1, which is the rate-limiting enzyme of the polyamine biosynthesis pathway that catalyses ornithine to putrescine, was up-regulated in TEI-SARM2-treated muscles, suggesting that polyamines, which are small, abundant, aliphatic molecules present in all mammalian cells, may play a role in skeletal muscle regulation. To determine the effect of polyamines, muscle growth was investigated using mouse C2C12 myoblasts treated with spermidine, a representative polyamine. After 48 h, the number of C2C12 cells was significantly increased (Figure 8).

**Discussion**

Muscle wasting is a frequent condition concomitant with disuse, cancer cachexia, sarcopenia, and frailty. Steroidal androgens such as ND have beneficial effects on muscle and bone but are not widely used due to safety concerns including cardiovascular events. SARMs, which bind to AR and display tissue-selective activation, present a preferable alternative. Ostarine has been evaluated in clinical studies for its efficacy in the prevention and treatment of muscle wasting in patients with cancer (POWER 1 and 2 trials, ClinicalTrials.gov ID: NCT01355484 and NCT01355497, respectively). In those studies, ostarine at 3 mg failed to show an increase in muscle strength.\(^{30}\) In contrast, testosterone at 600 mg/week for 5 months significantly increased muscle weight and muscle strength.\(^{30}\) Therefore, the development of SARMs that can achieve clinically relevant anabolic effects with comparable efficacy as those of testosterone is needed. TEI-SARM2, developed as an oral non-steroidal SARM, is a pharmaceutical candidate for the treatment of muscle wasting diseases because of its unique characteristics as follows. Interestingly, TEI-SARM2 showed N/C interaction activity similar to that induced by DHT and ND, a clinically available anabolic steroid, whereas ostarine showed minimal activity (Table 1). TEI-SARM2 significantly increased muscle weight, similar to ND treatment, but did not affect prostate and testis weights, which were significantly reduced by ND treatment, suggesting organ/tissue-selective effects of TEI-SARM2 (Figure 1). The androgenic effects caused by anabolic steroids may potentially lead to stimulatory effects on androgenic tissues. Therefore, the therapeutic range of these agents is limited. This study suggests that TEI-SARM2 has potential beneficial effects on a wide variety of clinical conditions due to its selectivity for anabolic tissues. In rodents, LA/BC are known as highly androgen-sensitive muscles. Indeed, LA/BC fibre size markedly decreases after castration and increases with androgen treatment, but fibre number remains unchanged. In contrast, androgen effects on limb muscle mass and function in animal models remain controversial. It was reported that administration of the testosterone derivative stanozolol to mice does not increase mass and force of limb muscle.\(^{31}\) Therefore, we evaluated the anabolic effects of TEI-SARM2 mainly focusing on perineal skeletal muscles in male in this study.

In a super-aging society, age-related muscle wasting and weakness, also known as sarcopenia and muscle atrophy, after hip fracture have become issues in recent years. We sought to evaluate the efficacy of TEI-SARM2 in a disuse muscle atrophy model using hindlimb-unloading female rats. Oral treatment of TEI-SARM2 for 2 weeks prevented rapid

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**Figure 8** Effect of spermidine on mouse C2C12 myoblasts. Cells were seeded in a 96-well plate at \(2 \times 10^3\) per well and cultured for 24 h in medium containing 10% foetal bovine serum. Spermidine at a final concentration of 6–2000 nM was added, and the cells were incubated for another 48 h. Cell proliferation was measured using CellTiter-Glo. Data are presented as means ± standard deviation (\(n = 6\) per group). ** and *** indicate \(P < 0.01\) and \(P < 0.001\), respectively, compared with the control, by Dunnett’s test.
Anabolic effects by AR ligand is related with polyamine signaling

Cancer cachexia is a devastating muscle wasting syndrome. It is commonly observed in 80% of cancer patients and is one of the primary causes of cancer-related morbidity and mortality. Cancer cachexia treatment with the two most investigated compounds, a ghrelin receptor agonist and ostarine, failed to improve physiological functions in clinical trials. However, in this study, TEI-SARM2 successfully prevented body weight loss and muscle and fat wasting but had no effect on tumour growth in a mouse model of cachexia induced by transplantation of human gastric cancer cells (Figure 3). Moreover, in a rat model of cancer cachexia induced by transplantation of Yoshida AH-130 hepatoma cells, TEI-SARM2 not only prevented body weight loss but also improved survival rate. These anabolic effects of TEI-SARM2 observed in two different cancer cachexia models suggest that TEI-SARM2 may become a therapeutic drug candidate for cancer cachexia, for which there is no adequate therapy. It is well known that female rodents were more sensitive for increasing body weight and muscle weight by androgen than male rodents. In fact, it was reported that female cachexia mice had reduced body weight and hindlimb muscle mass compared with female control mice but lacked changes in protein concentration and specific force. Male cachexia mice had reduced protein concentration and reduced specific force but lacked altered body weight and muscle mass. Therefore, we used female rodents for disuse and cancer cachexia models; however, it is needed to clarify the sex difference of AR function in skeletal muscle in our future studies.

As many as 32% of patients who die of cancer had a cardiac disorder. Cancer cachexia is associated with impaired cardiac function, caused by progressive loss of cardiac tissue, especially the left heart ventricle. It has been postulated that this may contribute to multiple organ failure. Heart failure (HF) itself, in the absence of any other diseases, is associated with cardiac cachexia. Because HF is an independent cause of cachexia, both cancer cachexia-induced cardiac atrophy and HF may be additional factors contributing to cancer cachexia and thereby exacerbating muscle wasting. Cancer cachexia drives atrophy of the myocardium, which results in cardiac hypertrophy. Therefore, cancer cachexia-induced HF is distinguished from ‘classical’ HF, characterized by left ventricular hypertrophy and dilatation. The heart is more susceptible to catabolic stimuli than is skeletal muscle. We showed that TEI-SARM2 dose dependently restored heart weight in a mouse model of cancer cachexia induced by transplantation of MKN45 human gastric cancer cells. At 10 mg/kg, TEI-SARM2 restored heart weight to the level of intact mice. Therefore, it is necessary to assess the effect of TEI-SARM2 on cardiac function in cancer cachexia.

The effects of TEI-SARM2 in non-human primates as well as rodents should be confirmed in a preclinical study. After 1 month of TEI-SARM2 treatment in monkeys, body weight and LBM were significantly increased more than 20% compared with the vehicle control group (Figure 4). Therefore, TEI-SARM2 may be a promising new treatment approach for muscle wasting diseases such as fracture-related bedrest-induced muscle atrophy, cancer-related cachexia, and sarcopenia.

Furthermore, we investigated the role of AR in skeletal muscle using mARKO mice treated with TEI-SARM2. The LA and BC muscle weights of mARKO mice were significantly lower than those of littermate control mice, and a further reduction in the muscle weight of mARKO mice was induced by ORX (Figure 5), suggesting that AR in myofibres is essential for maintenance of skeletal muscle mass. TEI-SARM2 and ND treatments ameliorated ORX-induced LA/BC muscle loss in both mARKO and control mice. Interestingly, TEI-SARM2 and ND did not affect hindlimb muscle weight in mARKO mice, whereas both improved grip strength (Figure 5). These phenomena lead us to hypothesize that AR agonists, such as TEI-SARM2 and ND, act indirectly via AR in extra-myoﬁbre tissues. It was reported that the Odc1 gene, which encodes ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, is directly regulated by AR in skeletal muscle myoblasts. RNA analyses revealed that Odc1 expression was up-regulated by TEI-SARM2 in both mARKO and control mice. Although its expression level was different between mARKO and control, the degree of difference was comparable with the result of muscle weights. Therefore, the polyamine signal was focused. The result of RNA-seq analysis revealed that Spermidine/Spermine N1-Acetyltransferase 1 was up-regulated by TEI-SARM2 in AR-independent manner (data not shown). It was previously reported that acetyl-CoA levels were elevated and proteins were acetylated in mitochondria when fuel was increased. Moreover, supplementation with acetylase-inhibiting polyamines counteracted the protein hyperacetylation. These results indicated that acetyl-CoA consumption by the TCA cycle in Caenorhabditis elegans prevents protein hyperacetylation and thereby protects mitochondrial function during early embryogenesis. Therefore, it can be hypothesized that androgens including TEI-SARM2 promote acetylation of polyamine for regulation of mitochondria followed by regulating skeletal muscle function. Further study will be needed to clarify this hypothesis. In contrast, Igf1 was up-regulated by TEI-SARM2 in both mARKO and control mice to the same extent. These results indicate that AR ligands induce expression of direct target genes, such as Odc1, via myofibre AR, as well as indirect genes, such as Igf1, by secondary effects via extra-myoﬁbre AR. In addition, integrative analyses of RNA-seq data revealed direct and indirect AR agonist effects

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on gene expression profiles (Figure 7). Regarding on the autophagy-related genes, LC3b was up-regulated by ORX only in control mice, not in mARKO mice. TEI-SARM2 promoted the expression of LC3b in AR-independent manner. On the other hand, Lamp1 and Lamp2 were down-regulated by ORX in both wild and ARKO mice. TEI-SARM2 promoted the expression of Lamp1 and Lamp2. TEI-SARM2 did not affect the expression of p62. These results indicated that autophagy could be influenced by TEI-SARM2/AR signalling directly and indirectly. The 20 genes identified as direct targets of TEI-SARM2 from these analyses, including myostatin, which is a well-known inhibitor of myoblast proliferation, Rps6ka2, which is related to the PI3K/Akt/mTOR pathway, and p21-activated kinase 1, which is related to cell growth and cell motility, were extracted. In addition, four genes, Odf3i2 (outer dense fibre of sperm tails 3 like 2), Kcnq4 (potassium voltage-gated channel modifier subfamily G member 4), Otog (otogelin), and Ilr1 (interleukin 1 receptor type 2), were up-regulated by TEI-SARM2 in a muscle AR-dependent manner. Odf3i2 is expressed in testes, but its expression in skeletal muscle is not well understood. Ilr1 is also known as CDC121b, a decoy receptor of the IL-1R family. It was reported that IL1L2 bound to and thereby inhibited IL-1α/IL-1β/IL-1 receptor antagonists. IL-4 prevents the role of IL-1 by promoting expression of Ilr1. One possibility is that up-regulation of Ilr1 by TEI-SARM2 via muscular AR might be related to muscle hypertrophy. Moreover, we detected six genes that were up-regulated by TEI-SARM2 in a muscular AR-independent manner (Figure 7), and there are several explanations for how these genes might be related to muscle hypertrophy. However, the precise mechanism still remains unknown. Further studies to determine the extra-myofibre AR functions that indirectly induce expression of these genes are needed.

There are limitations in this study. The readouts from all the experiments were not completely same. In addition, serum levels of endogenous testosterone and/or DHEA levels were not measured because of technical limitations for LC–MS/MS, which is considered to be more precise method to analyse steroid hormones.

Conclusions

TEI-SARM2, which was developed as a small molecule SARM with a non-steroidal chemical structure, is available orally. Daily and once-weekly dosing of TEI-SARM2 induced potent and selective anabolic effects on muscles in relevant animal models. Significant advantages of TEI-SARM2 over existing therapies have been suggested in preclinical studies. In diseuse muscle atrophy models, a selective anabolic effect of TEI-SARM2 was detected in muscle. TEI-SARM2 improved experimental parameters such as body and muscle weights without any effect on tumour volume or survival rate in cancer cachexia models. Moreover, TEI-SARM2 induced anabolic effects on skeletal muscle via AR in myofibres as well as other androgen target cells. In addition, our results suggest a relationship between polyamines and the anabolic effects of AR ligands.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 mRNA levels in the gastrocnemius muscle of female mice model of cachexia using MKN45 human stomach adenocarcinoma cells. (a) Atrogin-1, (b) MurF1, (c) FoxO1, (d) FoxO3a, (e) IGF1, (f) Odc1 mRNA BALB/c nu/nu female mice (6 weeks old) were transplanted with MKN45 human stomach adenocarcinoma cells and then administered oral TEI-SARM2 at 1 or 10 mg/kg daily for 4 weeks. ND at 10 mg/kg was administrated once-biweekly subcutaneously for 4 weeks. Gastrocnemius muscle were weighed after euthanasia. Data are presented as means ± SD (n = 6 per group). * and ** indicate p < 0.05, p < 0.01 and p < 0.001, respectively, compared with vehicle treatment, by Dunnett’s test. # indicates p < 0.001, compared with sham treatment, by Student’s t-test. Figure S2 mRNA levels in the BC muscle of mARKO mice. mARKO mice and WT littermates (14 weeks old) were orchietomized. On day 9 after orchietomy, the mice were treated with TEI-SARM2 (10 mg/kg, weekly, p.o.) or ND (100 mg/kg, one-time administration, s.c.) for another 14 days. The levator ani (LA) and bulbocavernosus (BC) muscles were collected after euthanasia on day 23. Srd5a1, st5, nc0a1 mRNA levels in BC muscle were measured by qRT-PCR. Data are presented as means ± SD (n = 4 per WT group, and n = 5, 6, 7 and 6 in
Anabolic effects by AR ligand is related with polyamine signaling from Teijin Ltd. and Teijin Pharma Ltd. All other authors declared no conflicts of interest.

Author contributions
M.K., K.Y., and Y.I. designed the experiments and wrote the manuscript. M.K. and all other authors performed the experiments.

Conflict of interest
M.K., K.N., and K.Y. are employees of Teijin Ltd. K.H. is an employee of Teijin Pharma Ltd. Y.I. received research support from an employee of Teijin Pharma Ltd. Y.I. received research support from an employee of Teijin Pharma Ltd. All other authors declared no conflicts of interest.

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