European Journal of Pharmacology 720 (2013) 107–114

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine Pharmacology

A novel selective androgen receptor modulator, NEP28, is efficacious in muscle and brain without serious side effects on prostate

Kazumasa Akita [a,*], Koichiro Harada [a], Junji Ichihara [b], Naoko Takata [a], Yasuhiko Takahashi [a], Koichi Saito [a]

[a] Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., Osaka, Japan
[b] Genomic Science Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan

ARTICLE INFO

Article history:
Received 7 May 2013
Received in revised form
18 October 2013
Accepted 23 October 2013
Available online 29 October 2013

Keywords:
Androgen
Selective androgen receptor modulator
SARM
Neprilysin
Amyloid

ABSTRACT

Age-related androgen depletion is known to be a risk factor for various diseases, such as osteoporosis and sarcopenia. Furthermore, recent studies have demonstrated that age-related androgen depletion results in accumulation of β-amyloid protein and thereby acts as a risk factor for the development of Alzheimer's disease. Supplemental androgen therapy has been shown to be efficacious in treating osteoporosis and sarcopenia. In addition, studies in animals have demonstrated that androgens can play a protective role against Alzheimer's disease. However, androgen therapy is not used routinely for these indications, because of side effects. Selective androgen receptor modulators (SARMs) are a new class of compounds. SARMs maintain the beneficial effects of androgens on bone and muscle while reducing unwanted side effects. NEP28 is a new SARM exhibiting high selectivity for androgen receptor. To investigate the pharmacological effects of NEP28, we compared the effects on muscle, prostate, and brain with mice that were androgen depleted by orchidectomy and then treated with either placebo, NEP28, dihydrotestosterone, or methyltestosterone. We demonstrated that NEP28 showed tissue-selective effect equivalent to or higher than existing SARMs. In addition, the administration of NEP28 increased the activity of neprilysin, a known Aβ-degrading enzyme. These results indicate that SARM is efficacious for the treatment of not only osteoporosis and sarcopenia, but also Alzheimer's disease.

1. Introduction

Androgens play vital roles in maintaining male phenotype (George and Wilson, 1986; Mooradian et al., 1987). Androgen supplementation has efficacy in osteoporosis, sarcopenia, frailty, and sexual dysfunction (Gooren, 2003; Wu, 1992). It is also effective in promoting increase in bone density and muscle mass in wasting diseases such as cancer and HIV (Bhasin et al., 1998). However, clinical use of androgens has been limited because of concerns about side effects (Bhasin and Bremner, 1997), including prostate hyperplasia and prostate cancer. Induction and progression of prostate cancer are especially serious side effects. Therefore, to use androgens safely and effectively in clinical practice, it is necessary to develop selective androgen receptor modulators (SARMs) that act only in tissues in which their effects are desired.

Existing SARMs maintain anabolic effect on bone and muscle while reducing unwanted effect on prostate (Miner et al., 2007; Piu et al., 2008; Vajda et al., 2009; Schmidt et al., 2010). However, the mechanism of tissue selectivity is still unclear. It is reported that tissue selectivity of compounds is determined by the interaction of ligand–androgen receptor complex and transcriptional coactivators. The interaction with transcriptional coactivators is necessary for androgen receptor to transcript down-stream genes, and SARMs alter the affinity of androgen receptor for transcriptional coactivators. A recent study has demonstrated that one SARM, LGD-2226, alters androgen receptor interaction with GRIP1 more readily than 5α-dihydrotestosterone (DHT) does (Miner et al., 2007). However, the precise coactivators responsible for tissue-selectivity have not been identified.

Recent studies have demonstrated that androgens have beneficial effects on Alzheimer's disease (Rosario and Pike, 2008; Rosario et al., 2006). Age-related androgen depletion is considered as the most significant risk factor for the development of Alzheimer's disease (Evans et al., 1989; Jorm et al., 1987; Rocca et al., 1986). In addition, recent experimental findings indicate that androgens reduce the level of amyloid β (Aβ), the protein widely implicated in the initiation of Alzheimer's disease pathogenesis. Circulating levels of testosterone are inversely correlated with plasma levels of Aβ in brain of aged men. Recently it was shown that androgens suppressed the accumulation of Aβ in brain by up-

* Correspondence to: Environment Health Science Laboratory, Sumitomo Chemical Co., Ltd., 1-98, Kasugadenaka 3-chome, Konohana-Ku, Osaka 554-8558, Japan. Tel.: +81 6 6439 9866; fax: +81 6 6466 5319.
E-mail address: akitak@sc.sumitomo-chem.co.jp (K. Akita).

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.10.042

regulating the expression of neprilysin (NEP) (Yao et al., 2008), an Aβ-degrading enzyme (Iwata et al., 2000; Saito et al., 2005). These results indicate that androgen therapy is useful for the treatment of Alzheimer's disease. Because of their side effects, androgens used clinically should be SARMs; however, there is little evidence regarding the action of SARMs in brain.

In this study, we have developed one non-steroidal SARM, NEP28. To investigate tissue selectivity of NEP28, we have assessed the efficacy in muscle and prostate using castrated rats. In addition, we have investigated the action of NEP28 in brain by evaluating the activity of NEP and the level of Aβ. Here we show that NEP28 may be useful for the treatment for various age-related diseases, including sarcopenia and Alzheimer's disease, without some of the side effects of current steroidal androgens.

2. Material and methods

2.1. Cell culture

CHP212 and Hela cells were cultured at 37 °C in Dulbecco's modified Eagle medium (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Nakarai). 22RV1 cells were cultured at 37 °C in RPMI-1640 medium (Wako) supplemented with 10% charcoal stripped fetal bovine serum (FBS) (Invitrogen), 100 units/ml; penicillin, and 100 μg/ml streptomycin (Nakarai). 17β-estradiol, DHT, and methyltestosterone (MT) were purchased from Wako. 3,3′,5-triiodothyronine, progesterone, aldosterone, and dexamethasone were purchased from Sigma.

2.2. Plasmid construction

cDNA encoding human androgen receptor (GenBank accession number NM_000044) was obtained by PCR from human prostate cDNA (Clontech). We constructed an expression plasmid for human androgen receptor, pRc/RSV-hAR, by inserting the corresponding cDNA sequence encoding human androgen receptor into the pRc/RSV vector (Invitrogen). The reporter plasmid pARE-Luc contains three androgen-responsive elements (ARE) $(TAGTAAAG\text{-}TACTCCAAGAACCTATTT)_3$ upstream of the minimum promoter region that drive expression of the firefly luciferase reporter gene. The pM cloning vector for expressing the GAL4 DNA-binding domain (DBD) fusion protein, pVP16, for expressing the VP16 transcriptional activation domain (VP16AD) were obtained from Clontech. A luciferase reporter vector, pG5luc, containing five GAL4-binding sites upstream of a minimal TATA box with the firefly luciferase gene was obtained from Promega. The ligand-binding domains (LBD) of human nuclear receptors, estrogen receptor (amino acids 249–595), androgen receptor (623–919), mineralocorticoid receptor (667–985), glucocorticoid receptor (485–777), thyroid hormone receptor (158–456), and progesterone receptor (631–933) were inserted into the *EcoRI* and *SalI* sites of pM for estrogen receptor, thyroid hormone receptor, and progesterone receptor, and *BamHI* and *SalI* sites of pM for androgen receptor, mineralocorticoid receptor, and glucocorticoid receptor; cloning receptors and their junctures were sequenced; and LBDs were determined to be correctly in-frame with GAL4DBD. The receptor-interacting domain (RID) of transcriptional intermediary factor 2 (TIF2) (amino acids 624–1287) was inserted into pVP16 digested with *EcoRI–BamHI* for the production of fusion proteins with the VP16AD. The sequence and reading frame of TIF2-RID were confirmed by DNA sequencing.

2.3. Luciferase assay

pRc/RSV-hAR and pARE-Luc were introduced into CHP212 cells and 22RV1 cells using TransIT-Neural Transfection Reagent or TransIT-Prostate Transfection Reagent (TAKARA) in accordance with the manufacturer's instructions. Subsequently, androgen receptor promoter activities in transfected cells were evaluated using Steady-Glo Luciferase Assay System kit (Promega).

2.4. Mammalian two-hybrid assay

Plasmids were introduced into Hela cells using Lipofectamine (Life Technologies) in accordance with the manufacturer's instructions. Luciferase activities were evaluated using Steady-Glo Luciferase Assay System kit (Promega).

2.5. Animals

Animal studies were conducted by DIMS Institute of Medical Science (Aichi, Japan). Twelve-week-old male Sprague–Dawley rats were purchased from Charles River Laboratories Japan. A 12 h light/dark cycle was maintained throughout the course of the experiment, with lights on at 7:00 AM. Animals were acclimated for a minimum of 7 days before any experimental procedures were performed. Standard laboratory rodent chow and water were provided *ad libitum*. Animals were anesthetized by ether anesthesia and sham-operated or orchidectomized (ORDX). Two weeks after surgery, all animals were implanted with a subcutaneous 21-day continuous-release ALZET osmotic pump (DURECT) containing either DHT, MT, NEP28, or vehicle. Doses of MT were determined based on the results of preliminary experiments (data not shown). Appropriate doses of DHT and NEP28 were estimated by considering *in vitro* activity in 22RV1 cells. Following 2 weeks of treatment, rats were killed and the ventral prostate, seminal vesicle, and levator ani muscle were collected. They were fixed in 10% neutral buffered formalin and then weighed. Brains were quickly harvested and stored at −80 °C. All experiments were performed in accordance with The Guide for Animal Care and Use of Sumitomo Chemical Co., Ltd. and DIMS Institute of Medical Science guidelines for animal experiments.

2.6. Measurement of Aβ amount

Quantification of brain Aβ40 and Aβ42 was performed using sandwich ELISA, Human/Rat beta Amyloid (40) ELISA Kit Wako II (Wako). Hemibrains were homogenized in 10 times volume of homogenization buffer (50 mM Tris–HCl [pH 8.0], 5 M Guanidine) and then incubated for 4 h at room temperature. After incubation, the homogenates were diluted 10 times with phosphate buffered saline (PBS) containing 5 mM ethylenediaminetetraacetate (EDTA) and mixed well. Ten microliters of diluted homogenates were used for measuring protein amount using BCA protein assay reagent kit (Pierce). Casein was then added to diluted homogenates. The mixtures contained a final concentration of 5% casein. The mixtures were centrifuged at 16,000*g* at 4 °C for 15 min. The supernatants obtained by centrifugation were subjected to ELISA.

2.7. Measurement of neprilysin activity

Hemibrains were homogenized in 5 times volume of homogenization buffer (10 mM HEPES-NaOH [pH 7.4], 250 mM Sucrose) supplemented with a protease inhibitor cocktail (Nakarai). The homogenates were centrifuged at 9000*g* at 4 °C for 15 min. The supernatants were then centrifuged at 200,000*g* at 4 °C for 20 min. The membrane fraction pellets were resuspended with 1 ml of assay buffer (50 mM HEPES-NaOH [pH 7.4], 0.05% Brij-35), and the