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Human expanded polyQ androgen receptor mutants in neurodegeneration as a novel ligand target

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Running Title: A novel function of liganded hAR Q mutant

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Abbreviations: AR, androgen receptor; SBMA, spinal and bulbar muscular atrophy; polyQ, polyglutamine; NR, nuclear receptor; C domain, conserved middle region; DBD, DNA binding domain; LBD, ligand binding domain; AF-1, autonomous activation function-1; H12, α-helix 12; SERM, selective estrogen receptor modulator; DHT, dihydrotestosterone; HF, hydroxyflutamide; BIC, bicalutamide; NIL, nilutamide; RU, 4-(4,4-dimethyl-2,5-dioxo-1-imidazolidinyl)-2-trifluoromethylbenzonitrile; SEM, scanning electron microscopy; DMSO, dimethyl sulfoxide; SAS, statistical analysis system; ARE, androgen response element; GMR, glass multimer receptor.

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Abstract

Androgen receptor (AR) plays key roles in various biological events, including pathological processes such as prostate cancer, androgen insensitive syndrome, and spinal and bulbar muscular atrophy (SBMA). SBMA is caused by mutation of the expanded polyglutamine (polyQ) stretches in the AR gene. Recently, we established a *Drosophila* SBMA model that expresses the expanded polyQ hAR mutant in eyes, which monitors neurodegeneration as a rough eye phenotype. In addition, we showed that androgen binding to the mutant hAR causes structural alterations, leading to the onset of neurodegeneration in the fly eyes. In the present study, we examined if the ligand-induced neurodegeneration via the hAR mutant is coupled with the known ligand-induced transactivation function of hAR. By testing several known AR antagonists and several of their structure-related compounds, we unexpectedly found that none of the AR ligands antagonized the hAR mutant neurodegeneration function, and surprisingly, compound RU56279 was more potent in inducing neurodegeneration. However, in vitro and in vivo mammalian assays showed that RU56279 exhibited the expected antagonistic activity with the same potency as those of the other compounds. Thus, these findings suggest the presence of a novel ligand-induced function of the polyQ hAR mutant in neurodegeneration that could not be prevented by known antagonists for the hAR transactivation function.

Introduction

Androgen plays pivotal roles in male reproductive organs and sexual behaviors (Mooradian et al., 1987; Wilson, 1999), and is also well known to be deeply involved in pathophysiological events like androgen-dependent prostate cancer development and androgen-induced onset of spinal and bulbar muscular atrophy (SBMA), which is a rare degenerative disease of motor neurons, characterized by progressive muscle atrophy and weakness in male patients, usually beginning at 30–50 years of age (La Spada et al., 1991; Choong and Wilson, 1998; Merry and Fischbeck, 1998). Most of such androgen actions in physiological and pathophysiological events are considered to mediate gene regulation by the nuclear androgen receptor (AR). Mapping studies and functional analyses of SBMA cases revealed expansions in the number of trinucleotide CAG repeats in the first exon of the AR gene, which generate expanded polyglutamine (polyQ) stretches in the N-terminal A/B domain of the hAR protein. Disease onset occurs when the stretches contain more than 40 glutamine residues, compared to a range of 8 to 34 glutamine residues in normal individuals(La Spada et al., 1991; Merry and Fischbeck, 1998).

AR is a member of the nuclear receptor (NR) superfamily and acts as a ligand-inducible transcription factor (Mangelsdorf et al., 1995; Glass and Rosenfeld, 2000). Members of the nuclear receptor superfamily share common structural features with distinct functional domains, referred to as domains A to E/F. The highly conserved middle region (C domain) acts as a DNA binding domain (DBD), while the ligand binding domain (LBD) is located in the less well-conserved C-terminal E/F domain. The LBDs of most nuclear receptors, including AR, have been analyzed and have been

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shown to consist of 12 α-helices that form a pocket to capture cognate ligands (Shiau et al., 1998; Poujol et al., 2000). The autonomous activation function-1 (AF-1) within the A/B domain is ligand-independent, while the AF-2 in the LBD is induced upon ligand binding (Kato et al., 1995). Ligand-free LBD appears to suppress the function of AF-1, while ligand binding to the LBD is thought to evoke the function of LBD and to restore the A/B domain function through an as yet undescribed intramolecular alteration of the entire receptor structure. During ligand-induced transcriptional controls, AF-1 and AF-2 act as interacting regions for the coregulators (He et al., 1999; Watanabe et al., 2001; Shang et al., 2002). Upon ligand binding, the most C-terminal α-helix 12 (H12) in the LBD shifts position to create a space, with helices 3 to 5 serving as the key interface following dissociation of corepressor complexes and association of coactivator complexes (Freedman, 1999; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Yanagisawa et al., 2002). The angle of H12 shifting in the Type I NR is believed to be dependent on the features of ligands, generating tissue-specific actions of synthetic ligands like SERM through ligand-specific recruitment of coregulators and complexes (Brzozowski et al., 1997).

In a previous report, we had established a *Drosophila* SBMA model by introducing the expanded polyQ hAR gene, and showed that like the other expanded polyQ mutant human proteins expressing in *Drosophila* eyes, the hAR polyQ mutant AR caused an SBMA neurodegenerative phenotype, rough eye, in an androgen-dependent manner (Takeyama et al., 2002). The molecular basis that the ligand-bound hAR polyQ mutants cause neurodegeneration in human brains as well as fly eyes still remains elusive, and androgen responsiveness might be different between

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fly eyes and human brains. However, considering that the onset of the neurodegeneration is caused by androgen binding to the hAR polyQ mutants, together with the observations that the structural alterations of most NR LBDs by ligand binding depend on the ligand type (Brzozowski et al., 1997), utilizing this fly SBMA model we can assess if the ligand-induced neurodegeneration via the hAR polyQ mutant is coupled with the transactivation function.

To test this idea, the present study was undertaken to test if known hAR antagonists and their structure-related compounds exhibit the expected antagonistic activity in the *Drosophila* SBMA model. Surprisingly, the known antagonists failed to inhibit the ligand-induced neurodegeneration in the fly eyes. Among the tested ligands, RU56279 was found as the most potent inducer of the SBMA phenotype in our model. However, we could confirm the expected antagonistic activities of these compounds in mammalian systems. Thus, these findings suggest the presence of a novel ligand-induced function of the polyQ hAR mutant in neurodegeneration that could not be prevented by known antagonists for the hAR transactivation.

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Methods

Chemicals

Dihydrotestosterone (DHT) was purchased from Fluka AG (Buchs, Switzerland). [^{3}H]-mibolerone was purchased from Amersham (Little Chalfont, England). Testosterone Propionate was purchased from Nakarai Tesque (Kyoto, Japan). Hydroxyflutamide (HF), bicalutamide (BIC), nilutamide (NIL), 4-(4,4-dimethyl-2,5-dioxo-1-imidazolidinyl)-2-trifluoromethylbenzonitrile (RU56279, RU), and their structure- related compounds; *N*-[4-cyano-3-(trifluoromethyl)phenyl]-2-hydroxy-2-methyl-3-(pyridin-2-ylthio)propan amide, *N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-(ethylsulfonyl)-2-hydroxy-2-methylpropanami de, *N*-[4-cyano-3-(trifluoromethyl)phenyl]-2-hydroxy-2-methylpropanamide, 2-{[4-cyano-3-(trifluoromethyl)phenyl]amino}-1,1-dimethyl-2-oxoethyl acetate, 4-(5-imino-3,4,4-trimethyl-2-thioxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile, 2-(trifluoromethyl)-4-(3,4,4-trimethyl-5-oxo-2-thioxoimidazolidin-1-yl)benzonitrile, 4-[3-(4-hydroxybutyl)-5-imino-4,4-dimethyl-2-thioxoimidazolidin-1-yl]-2-(trifluoromet hyl)benzonitrile, 4-(5-imino-4,4-dimethyl-2-oxoimidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile, and 2-(trifluoromethyl)-4-(3,4,4-trimethyl-2,5-dioxoimidazolidin-1-yl)benzonitrile were synthesized at Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan).

Drosophila Stocks and Generation of Transgenic Flies

All general fly stocks and the ptc-GAL4 line were obtained from the Bloomington *Drosophila* Stock Center. Transgenic constructs together with pπ 25.7 wc transposase were microinjected into 5–30 min old w1118 embryos reared at 18°C, using a micromanipulator (Leica). Several transgenic lines were generated (Tsuneizumi et al., 1997). The AR mutant cDNAs in pCaSpeR3 and an ARE-GFP reporter construct (GFP-TT in pCaSpeR3 with a consensus ARE in its promoter) were specifically constructed for microinjection into *Drosophila*. Plasmid rescue and sequencing were performed to confirm the presence of AR mutants in the transgenic lines. Target chromosomes were separated from those carrying the GAL4-driver by crossing with flies harboring second and third balancer chromosomes CyO and TM3, respectively. The GMR-GAL4 line, expressing GAL4 in the retina driven by the glass multimer reporter, was utilized as the GAL4-driver line (Moses and Rubin, 1991). The UAS-Q127 lines were the generous gift of Dr. Kazemi-Esfarjani (Kazemi-Esfarjani and Benzer, 2000).

Immunofluorescence and Histology

Tissues were dissected and fixed for 20 min in 4% formaldehyde (Tanimoto et al., 2000) and incubated with a primary antibody, hAR (N-20), that recognized the N-terminal A/B domain of AR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cy5-conjugated AffinityPure donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as the secondary antibody for immunofluorescence staining. Confocal microscopy was performed with a Zeiss confocal laser scanning system 510 (Zeiss, Oberkochen, Germany). For scanning electron microscopy (SEM) images, whole flies were dehydrated in ethanol,

critical-point dried, and analyzed with a JSM 5400 microscope (JEOL, Tokyo, Japan).

Western Blot Analysis

To detect hAR and GFP expression in *Drosophila*, cell lysates from the heads of adult eyes or third instar larvae with or without ligand were separated by 7.5% SDS-PAGE and detected with hAR (N-20) antibody and GFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and expression levels measured using Adobe Photoshop software facility. Fold activation of hAR in *Drosophila* was shown as GFP expression signal intensity normalizing with hAR expression signal intensity.

Binding Assay for Rat Androgen Receptor

The ventral prostate gland was obtained from twenty-week-old male Wistar rats 24 hours after castration. The homogenized tissue was spun at 800g for 20 minutes. Next, the supernatant was subjected to further centrifugation at 223,000g for 60 minutes, and the resulting supernatant was recovered to obtain the cytosol fraction. The cytosol fraction was adjusted to a protein concentration of 1 mg/ml and used as a rat androgen receptor solution. [^{3}H]-mibolerone, triamcinolone acetate (Sigma Chemical Co., St. Louis, MO), and dimethyl sulfoxide (DMSO, Nakarai Tesque, Kyoto, Japan) were added to 400 µl of the rat androgen receptor solution to final concentrations of 1 nM, 1 µM, and 5%, respectively, and the final volume was adjusted to 0.5 ml. After 18 hours at 4ºC, this solution was mixed with 500 µl of a solution containing 0.05% of Dextran-T70 (Amersham, Little Chalfont, England) and 0.5% of Darco G-60 (Wako Pure Chemical Industries, Osaka, Japan). This mixture was incubated at 4ºC for 15

minutes and then subjected to centrifugation at 1500 g for 15 minutes to recover the supernatant. A 600 µl portion of the recovered supernatant was mixed with 5 ml of Aquasol-2 (Perkin-Elmer, Boston, MA), then the radioactivity was measured to calculate the total amount of [^{3}H]-mibolerone that bonded to the rat androgen receptor. The amount of non-specific binding was calculated in the same manner by adding a DMSO solution containing unlabeled mibolerone at a final concentration of 40 µM. The difference between the total binding amount and the non-specific binding amount was defined as the specific binding amount. The specific binding amount of [^{3}H]-mibolerone bound to the rat androgen receptor in the presence of a compound was calculated by adding a DMSO solution containing various concentrations of the compound, simultaneously with [^{3}H]-mibolerone, and carrying out a similar reaction as described above. The IC50 value of the inhibition activity of the compound on the specific binding of [^{3}H]-mibolerone was obtained by nonlinear analysis using SAS (statistical analysis system). Also, the dissociation constant Ki was calculated from the IC50 value by the formula of Cheng and Prusoff (Cheng and Prusoff, 1973).

Evaluation of Transcriptional Activity for the Human Androgen Receptor

CHO cells were transfected at 40-70% confluence in 10-cm petri dishes with a total of 20 µg hAR expression and reporter plasmids (pMAMneoLUC, MMTV-luciferase reporter plasmid, BD Biosciences Clontech, Palo Alto, CA; and pSG5-hAR, human androgen receptor expression plasmid; or SV40-LUC, SV40-luciferase reporter plasmid containing the neomycin resistant gene) by calcium phosphate mediated transfection (Furutani et al., 2002; Kinoyama et al., 2004). The

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transfected cells were selected in the culture medium supplemented with neomycin. The stable transformants that had high expression of hAR were designated as AR/CHO#3 or SV/CHO#10, respectively (Furutani et al., 2002).

The AR/CHO#3 or SV/CHO#10 cells were plated onto 96-well luminoplates at a density of 20,000 cells/well. Four to eight hours later, the medium was changed to the medium containing DMSO, 0.3 nM of DHT, or 0.3 nM of DHT and a compound. At the end of the incubation the medium was removed, then the cells were lysed with 20 µl of lysis buffer [25 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-cyclohexanediamine-tetraacetic acid, 10% glycerol, and 1% TritonX-100]. Luciferase substrate (20 mM Tris-HCl (pH 7.8), 1.07 mM $(MgCO_3)4Mg(OH)2.5H_2O$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.47 mM luciferin, and 0.53 mM ATP) was added and luciferase activity was measured with an ML3000 luminometer (Dynatech Laboratories, Chantilly, VA).

Yeast Two-Hybrid System and ß-Galactosidase Assay

The pGBT9(GAL4-DBD)-AR(EF) fusion plasmid was constructed by inserting human AR-EF regions into the pGBT9 vector (BD Biosciences Clontech, Palo Alto, CA). ARA70 cDNA was inserted into pGAD10 (BD Biosciences Clontech, Palo Alto, CA), which included a GAL4 transactivation domain, to construct pGAD-ARA70. The pGBT9(GAL4-DBD)-AR(EF) plasmid was co-transformed with pGAD-ARA70 into *Saccharomyces cerevisiae* Y153 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3 leu2-112 URA3::GAL HIS3) by the lithium acetate method. Transformants were plated in medium lacking leucine and tryptophan and were grown overnight in 2 ml of

SD medium lacking leucine and tryptophan. These samples, diluted to an optical density at 600 nm of 0.02, were cultured overnight with compounds. Cells were then harvested and assayed for ß-galactosidase activity as described previously (Takeyama et al., 1999).

GST Pull-Down Assay

Human AR A/B domain (AF-1) and its Q52 mutant (Q52 AF-1) were expressed as GST fusion proteins [GST-AR(AF-1) and GST-AR(Q52 AF-1), respectively] in *E. coli*, as previously described, and bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ). The ^{35}S-labeled AR deletion mutant together with DNA and ligand binding domains CDE/F were incubated with beads bound with either GST-AR(AF-1) or GST-AR(Q52 AF-1) in the absence or presence of 10^{-6} M RU in NET-N buffer [0.5% Nonidet P-40, 20 mM Tris-HCl(pH 7.5), 200 mM NaCl, 1 mM EDTA] with 1 mM PMSF. Bound proteins were separated by 9% SDS-PAGE and lightly stained with Coomasie brilliant blue to verify equal protein loading and then visualized by autoradiography.

Antiandrogenic Activity in Castrated Immature Male Rats

Male Wistar Rats (Charles River Japan, Yokohama, Japan) weighing 75 to 90 g were used. The animals were given ordinary laboratory food and tap water ad libitum and housed under artificial light for 13 h/day (from 7:30 AM to 8:30 PM). All experiments were performed in compliance with the regulations of the Animal Ethical Committee of Yamanouchi Pharmaceutical. The rats were castrated and administered

orally either compounds or vehicle, and subcutaneously either vehicle or testosterone propionate for 5 consecutive days. The day following the last administration, the rats were weighed and necropsied. The ventral prostate of each rat was excised and weighed.

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Results

None of the known hAR antagonists and their derivatives could block the ligand-induced neurodegeneration in the fly SBMA model.

We had previously established the *Drosophila* SBMA model by expressing a human androgen receptor (hAR) gene containing expanded polyglutamine stretches (52 residues) [hAR(Q52) line] in fly eyes by the GAL4-UAS system (Takeyama et al., 2002). In this fly line, which also carries an exogenous GFP reporter gene with a consensus androgen response element (ARE) in the promoter (Fig. 1A), hAR(Q52) is ectopically expressed in eye neurons by a glass multimer receptor (GMR) gene promoter. The expression of hAR(wt) and hAR(Q52) protein, the construct of which were shown in Fig. 1B, was confirmed by immunohistochemistry with a specific hAR antibody and appears as red (Fig. 1C). The androgen [dihydrotestosterone, (DHT)] response in hAR(Q52) in the fly eyes was observed by GFP expression and appears as green, like in the wild-type hAR expressing fly eyes (see Fig. 1C). Expression of hARs and GFP proteins in eyes were further confirmed by Western blotting in total eye extracts (Fig. 1D, E). Using this model, we evaluated several hAR known antagonists and several of their structure-related compounds. In the flies expressing wild type hAR [hAR(wt) line], neither phenotypic abnormalities (representative data by DHT and RU are shown in Fig. 2A) nor significant GFP expression (quantitative data of representative observations of AR, GFP protein expressions by BIC, HF, NIL, RU were shown in Fig. 2B) was induced by the tested compounds. The compounds were then ingested by the hAR(Q52) line together with or without dihydrotestosterone (DHT).

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Similar responses to DHT and synthetic ligands as monitored as GFP expressions, which monitors the transactivation function of wild-type hAR, were observed in the hAR mutant (Fig. 2D). When ingested together with DHT, however, no compounds failed to antagonize the DHT action to induce the rough eye phenotype (Fig. 2C). Furthermore, surprisingly all of the known AR antagonists and the structure-related compounds alone were capable of inducing the rough eye phenotype in the hAR(Q52) line (Fig. 3A). Through a light microscope (LM) and scanning electron microscope (SEM), the eyes of the hAR(Q52) line that ingested these compounds had reduced ommatidia and lost pigmentation, which are typical neurodegenerative phenotypes (Fig. 2C and 3A). While all the compounds antagonized the DHT action to induce the transactivation of hAR mutant (Fig. 2D). It is notable that the content of hAR proteins in the eyes of the hAR(Q52) and the hAR(wt) lines appear unchanged after compound ingestion (Fig. 2B, D). However, we could not exclude a possible difference in ligand response between fly eyes and human tissues.

Among the tested compounds, we found that 4-(4,4-dimethyl-2,5-dioxo-1-imidazolidinyl)-2-trifluoromethylbenzonitrile, previously designated as RU56279 (Cousty-Berlin et al., 1994), induced the rough eye phenotype of hAR(Q52) more potently than any other tested compound (Fig. 2C and 3A). RU56279 is a structure-related compound of nilutamide that is also used as a hAR antagonist for clinical treatment of prostate cancer. To address if the effect of RU56279 to induce the rough eye phenotype mediates the hAR polyQ mutant, we examined the RU56279 effects on the eyes in wild-type flies and the transgenic fly expressing a 127 polyQ protein that develops rough eyes without any AR ligand treatment (127Q line)

(Kazemi-Esfarjani and Benzer, 2000). The rough eye phenotype of the 127Q flies was not enhanced by RU56279 (Fig. 3B), and RU56279 exhibited no action in the parent wild type fly line (GMR-GAL4). Together with the inability of RU56279 to induce neurodegeneration in the eyes of hAR(wt), the RU56279 effect appeared to mediate the polyQ hAR mutant.

RU56279 antagonized the DHT-induced transactivation function of hARs in mammalian systems.

Although RU56279 was reported as a metabolite of RU56187 with antiandrogenic activity in the rat model (Cousty-Berlin et al., 1994), its characterization as a hAR ligand, including in vitro evaluation, remained to be investigated. A binding assay for hAR showed that RU56279 binds to AR in the nanomolar range, with a Ki value of 34.2 nM (Fig. 4A). Next, we examined whether RU56279 acts as a hAR agonist or antagonist using CHO cells stably expressing the hAR vector together with an MMTV-luciferase reporter construct. RU56279 inhibited the DHT-induced transcription in a dose-dependent manner, while RU56279 alone did not stimulate transcription (Fig. 4B). Such RU56279 antagonistic actions to transiently expressed hAR were also observed in HeLa, Cos1 and 293F cells (data not shown). Next, the RU56279 effect on the ligand-induced interaction of hAR and ARA70, which is a reported coregulator protein of hAR as a direct interactant, in a yeast two hybrid system was examined (Yeh and Chang, 1996). RU56279 did not induce the interaction of ligand-bound hAR with ARA70 in yeast, while DHT binding could induce the interaction (Fig. 4C). While RU56279 disrupted the DHT induced interaction of the

hAR and ARA70 in a dose dependent manner as well as other known antagonists. Moreover, ligand-induced alterations of the hAR structure were directly analyzed using a GST pull-down assay. As shown Fig. 4D, RU could not induce interactions between A/B (AF-1) and E/F (AF-2) domains for hAR(wt) and hAR(Q52). The results of RU56279 shown in this experiment were similar to that of HF as reported previously (Takeyama et al., 2002). Finally, to test the RU56279 antagonistic activity in androgen-dependent prostate development, RU56279 was administered to castrated rats that were supplemented with testosterone propionate. RU56279 antagonized the testosterone action in prostate growth (Fig. 4E). Together with the known antagonists exhibiting the expected actions to antagonize the androgen actions in mammalian systems (data not shown), these findings suggest that RU56279 is an androgen antagonist in mammalian systems.

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Discussion

Drosophila as a transgenic animal model to study human steroid hormone receptors.

AR belongs to the NR gene superfamily, and acts as a ligand-inducible transcription factor. Since AR is believed to play a central role in androgen signaling pathways, any malfunction of AR tends to cause certain disorders. The physiological and pathological impacts of AR could be tested in a mouse model by disrupting the AR gene in a given tissue and overexpressing the gene transgenically (Chatterjee et al., 1996; Kawano et al., 2003; Sato et al., 2003; Sato et al., 2004). However the AR mutants, like the ones with expanded polyQ residues, are not easily studied through mouse genetics due to the time required for the identification of the coregulator responsible for hAR function and screening of a novel ligand to restore the impaired AR functions. We have established a number of fly lines expressing hARs and other mammalian steroid hormone receptors, and found that ectopic expression of these NRs is quite safe for fly life, even in the presence of the cognate hormone (Takeyama et al., 2002; Ito et al., 2004; Kouzmenko et al., 2004). The major reason for this safety may be explained by mammalian exogenous steroid hormone receptors binding to exogenous DNA as a homodimer, and therefore not competing for endogenous DNA binding sites for endogenous fly NR heterodimers, as the DNA elements recognized between NR homodimers and heterodimers are distinct (Mangelsdorf et al., 1995; McKenna and O'Malley, 2002).

No known hAR antagonist could block the ligand-induced neurodegeneration in fly eyes by the hAR polyQ mutant.

In our previous report, we showed in fly eyes that the onset of neurodegeneration caused by the hAR polyQ mutants is dependent on DHT binding with structural alterations (Takeyama et al., 2002). Since the ligand-independent function of the polyQ-included A/B domain in the ΔLBD polyQ hAR mutants is potent enough to induce the rough eye phenotype, we presume that the ligand-induced exposure of the polyQ repeats in the hAR A/B domain, which is apparently masked by unliganded hAR LBD, is a critical trigger step that initiates neurodegeneration. Therefore, for preventing the onset of neurodegeneration in SBMA patients, developing a novel hAR ligand not to induce the A/B domain exposure after the ligand-induced structural alteration is mandatory. Though we could not exclude a possible difference in the AR ligand response between human neurons and fly eyes, the neurodegenerative fly eyes have been applied as human models for hereditary disease caused by unusual expansions of polyQ, and the fly eyes phenotype by hAR polyQ mutants was undiscriminative at histological and biochemical levels from those by the other polyQ mutants.

In the present study, we evaluated AR antagonists and structure-related compounds using the hAR(Q52) *Drosophila* line as an SBMA model. Surprisingly, no compounds were able to antagonize the DHT-induced rough eye phenotype and among them, RU56279 was found as the most potent to induce neurodegeneration. Clearly, these compounds appear potent to induce structural alterations of the A/B domain , though they expectedly acted as antagonists on the hAR trasnsactivation function in the

mammalian systems as well as in the fly eyes. It is also likely that RU56279 is a novel AR ligand, which alters the structure of the hAR mutant in a manner different from the other AR ligands, though it remains unclear about the molecular basis of the difference in the ligand-induced structural alterations among the hAR ligands.

A novel mechanism of ligand-induced neurodegeneration by the hAR polyQ mutant.

The molecular mechanism of neurodegeneration by expanded polyQ proteins remains elusive, and recently the cellular aggregates of the polyQ mutant fragments have been shown as a protective response for cell death (Arrasate et al., 2004). Unlike the other polyQ mutants, the hAR mutant neurodegenerative function is ligand-inducible, though the neuronal abnormality through the expanded polyQ residues looks indistinguishable among the polyQ mutants. Ligand-induced alteration of the hAR mutants is presumed to trigger such pathological processes in neurons; however, any coregulators responsible for the pathological function of the hAR mutants are unknown. Since ligand-induced transactivation of the wild-type hAR as well as the hAR polyQ mutants is believed to require a number of transcriptional coregulators and complexes, it is possible to speculate that the ligand-bound hAR polyQ mutants either recruit a critical initiator for neurodegeneration or dissociate from a protective factor. Moreover, it may be possible to identify such facotrs using and investigating the hAR antagonists especially RU56279. In any case, identification of such a factor is required for revealing the molecular mechanism of the androgen-induced neurodegeneration via hAR polyQ mutants, and this fly SBMA model should be powerful for the genetic

screening of the coregulators (Takeyama et al., 2004). Most notably, a novel class of ligand may be developed based on inhibition of physical and/or functional interaction of the hAR polyQ mutants with the identified factor, and such idea should be addressed in human SBMA patients.

The present study clearly suggests that the hAR(Q52) fly lines are a novel tool to screen a new class of hAR synthetic ligands, particularly the antagonist for the hAR polyQ mutants in neurodegeneration suffered in SBMA patients.

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Footnotes

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Legends for figures

Figure 1. Ectopic expression of functional human androgen receptors in *Drosophila* eyes.

(A) Expression of human AR proteins in *Drosophila* eyes using the *GAL4-UAS* system. To monitor the ligand-induced transactivation of hAR proteins, hAR-expressing flies are further crossed to flies carrying a GFP reporter gene. GFP expression was induced by ligand –bound hAR that binds a the concensus androgen responsive element (ARE) in the GFP receptor gene promoter.

(B) Human AR constructs.

Location of the polyglutamine region (red boxes) in relation to the DNA binding domain (black boxes, C domain). Transactivation function 1 region is localized within the N-terminal A/B domain, and transactivation function 2 region is localized within C-terminal E/F domain.

(C) Ligand-induced transactivation of hARs in eye discs. Expression of hAR in third instar larva eye discs driven by *GMR-GAL4* was detected with hAR antibody (N-20) (red). Transactivation of hAR was estimated by GFP expression (green). (D) Human AR and GFP expression in four pairs of total adult heads as detected by Western blotting. (E) Fold activation was calculated using hAR expression levels as normalizing factor. Dihydrotestosterone (DHT) was added at 10^{-5} M in fly diet during larval stage for ingestion.

Figure 2. Agonist-induced rough eyes in the fly line expressing human polyQ AR mutant.

(A) Effect of ligands on the eyes of hAR(wt) fly line. The pannels showed the light microscopic (LM) and scanning electron microscopic (SEM) images of adult eyes of 5-day-old flies treated with the indicate ligands.

(B) Effect of ligands on transactivation of wild type hAR in eye imaginal discs. The pannels showed the hAR and GFP expressions in four pairs of adult eyes at 5-day-old. Ligands [10^{-5} M dihydrotestosterone (DHT), bicalutamide (BIC) hydroxyflutamide (HF), nilutamide (NIL), or RU56279 (RU)] were treated during larval stage. Fold activation by ligands was calculated using hAR expression levels as a normalizing factor.

(C) Known antagonists were unable to attenuate the androgen-induced rough-eye phenotype in the hAR(Q52) fly line. Light microscopic (LM) images of adult eyes at 5-day-old flies treated as the indicated ligands.

(D) Effect of ligands on transactivation of mutant hAR in eye imaginal discs. The panels showed the mutant hAR and GFP expression in four pairs of adult eyes at 5-day-old flies. Fold activation was calculated using hAR expression levels as a normalizing factor .

Figure 3. Antagonist-induced rough eyes in the fly line expressing human polyQ AR mutant.

(A) Known antagonists alone were potent to induce neurodegeneration of the hAR(Q52) fly eyes. Light microscopic (LM) and scanning electron microscopic (SEM) images of adult eyes of 5-day-old flies treated with ligands during larval stage were showed.

(B) No additive action of RU56279 in the rough eye phenotype of the Q127 fly line. Light microscopic (LM) and scanning electron microscopic (SEM) images of adult eyes from 5-day-old flies treated as larva with RU56279 (RU) were shown. Genotype is UAS-127Q in trans to GMR-GAL4 and GMR-GAL4.

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Figure 4. Anti-androgenic actions of the known antagonist RU56279 in in vitro and in vivo mammalian systems.

(A) Competitive binding of androgen antagonists with an agonist in the rat prostate cytsols. The rat prostate cytosols were incubated with unlabeled mibolerone (MIB), bicalutamide (BIC), or RU56279 (RU) at the indicated concentrations with 1 nM [^{3}H]-mibolerone (a hAR agonist). The radioactivity was measured as described in the Materials and Methods. Data are expressed as duplicate determinations.

(B) RU56279 as a hAR antagonist for hAR transactivation function. The stable transformant of CHO cells, which contain the human AR gene and MMTV-luciferase reporter gene (Furutani et al., 2002), were treated with either bicalutamide (BIC) or RU56279 (RU) at the indicated concentrations in the presence or absence of dihydrotestosterone (DHT) at 0.3 nM. After 18 hours, cells were harvested and assayed for luciferase activity as described in the Materials and Methods. Data are expressed as the mean ± SEM of triplicate determinations.

(C) RU56279 inhibited the androgen-induced interaction of human AR with a hAR cofactor in the yeast two-hybrid system. pGBT9(GAL4-DBD)-AR(EF) fusion protein and pGAD10(GAL4-AD)-ARA70 fusion protein (Yeh and Chang, 1996) were expressed in yeast containing the lacZ gene controlled by the GAL4 enhancer. The yeast cells were treated with either bicalutamide (BIC) hydroxyflutamide (HF), nilutamide (NIL), or RU56279 (RU) at the indicated concentrations in the presence or absence of dihydrotestosterone (DHT). Interaction of hAR with the cofactor was assessed by measuring ß-galactosidase activity. Data are expressed as the mean ± SEM of triplicate determinations.

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(D) Effects of RU56279 on interaction of hAR(AF-2) and hAR(Q52 AF-1) *in vitro*. Interaction was assessed by incubating a GST fusion protein with either hAR(AF-1) [GST-hAR(AF-1)] and mutant hAR(AF-1) with Q52 [GST-hAR(Q52 AF-1)] with *in vitro* translated [^{35}S]methionine-labeled hAR LBD [hAR(AF-2)] by pcDNA3-hAR 560-919.

(E) RU56279 on rat prostate growth as an androgen-antagonist. Male Wistar rats were castrated, and then treated with testosterone propionate along together with the indicated antagonists daily for 5 days. The rats were sacrificed and ventral prostates were removed and weighed. Data are expressed as the mean ± SEM obtained from 5 rats.

Figure 1

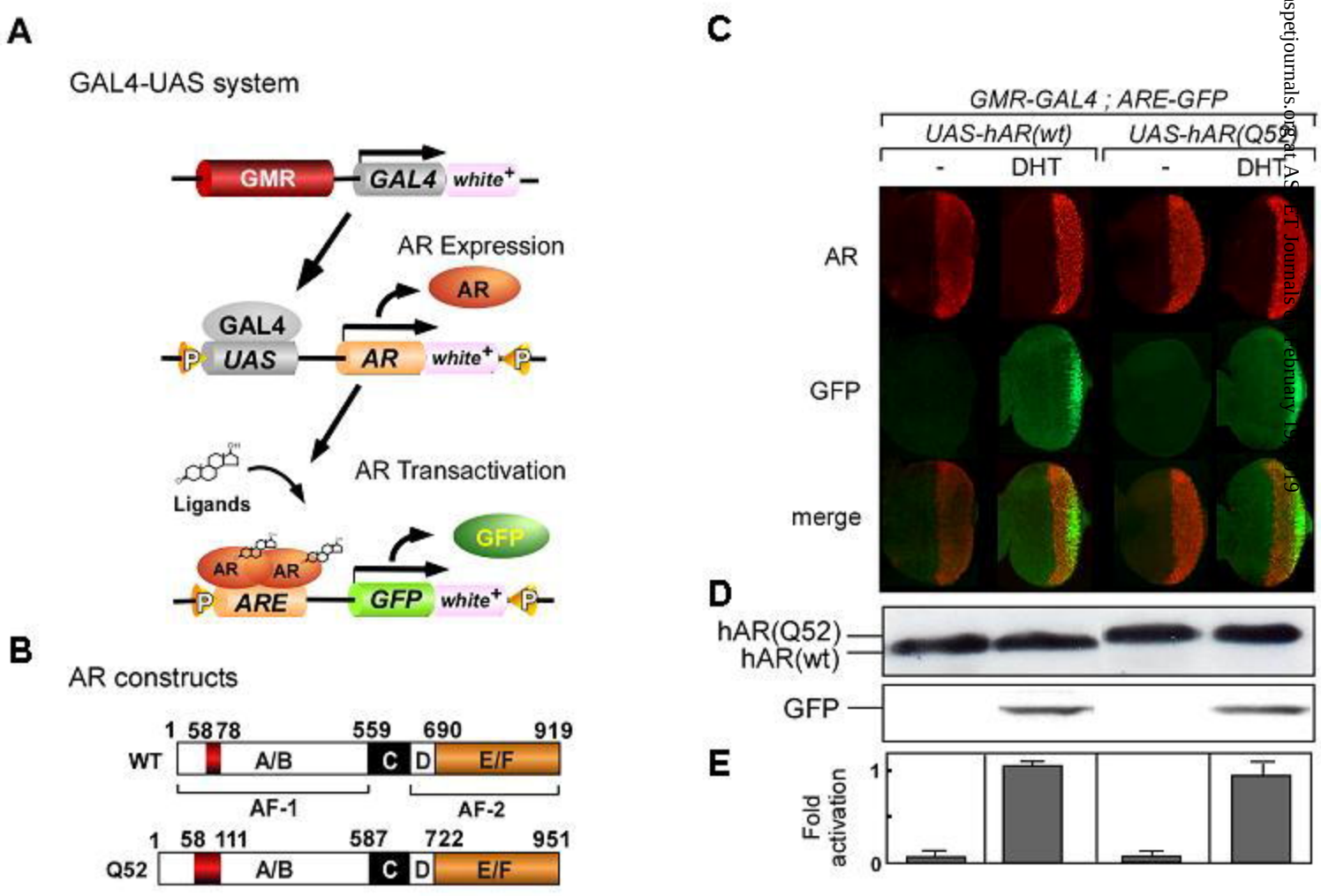

Figure 2

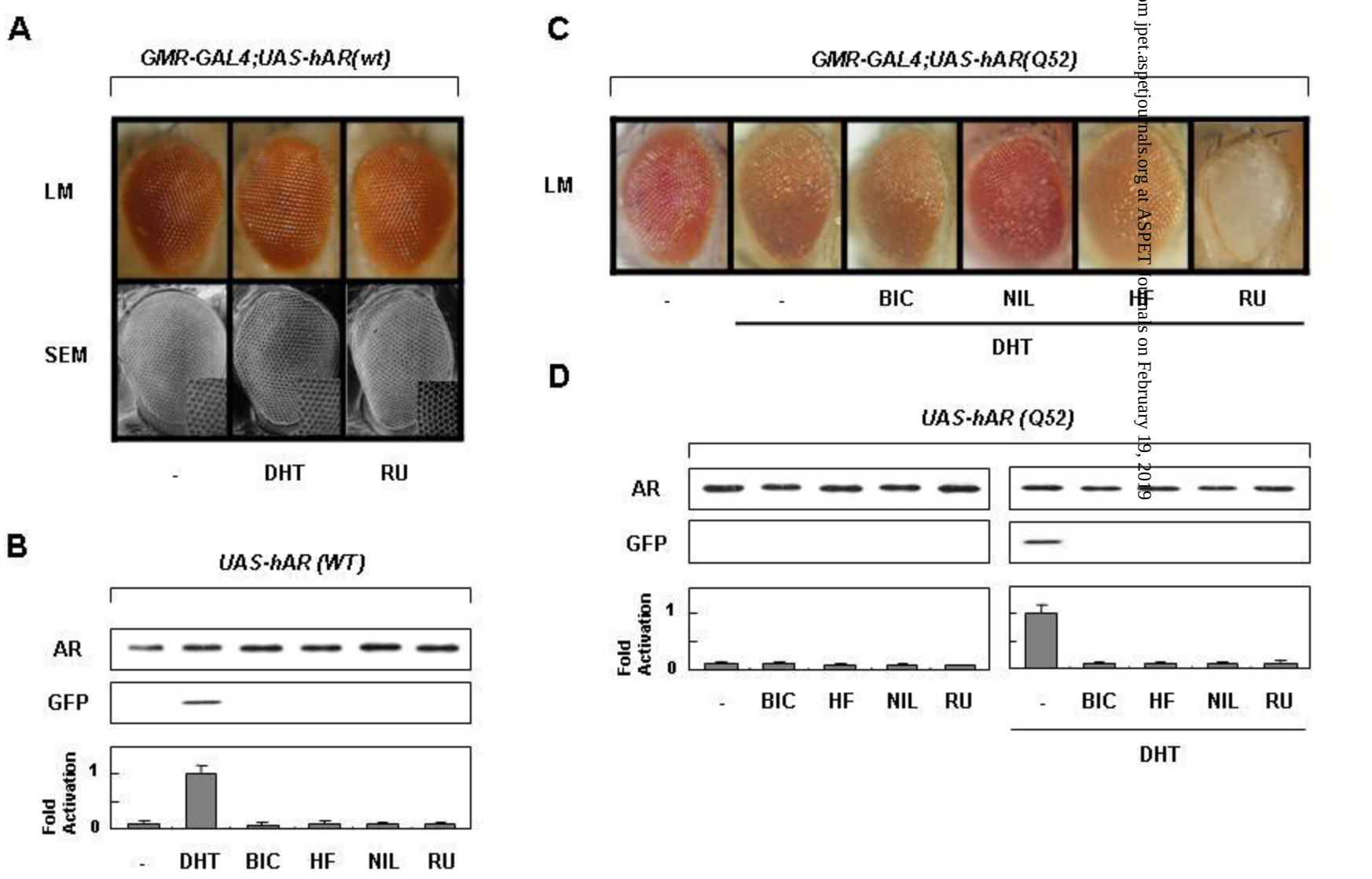

Figure 3

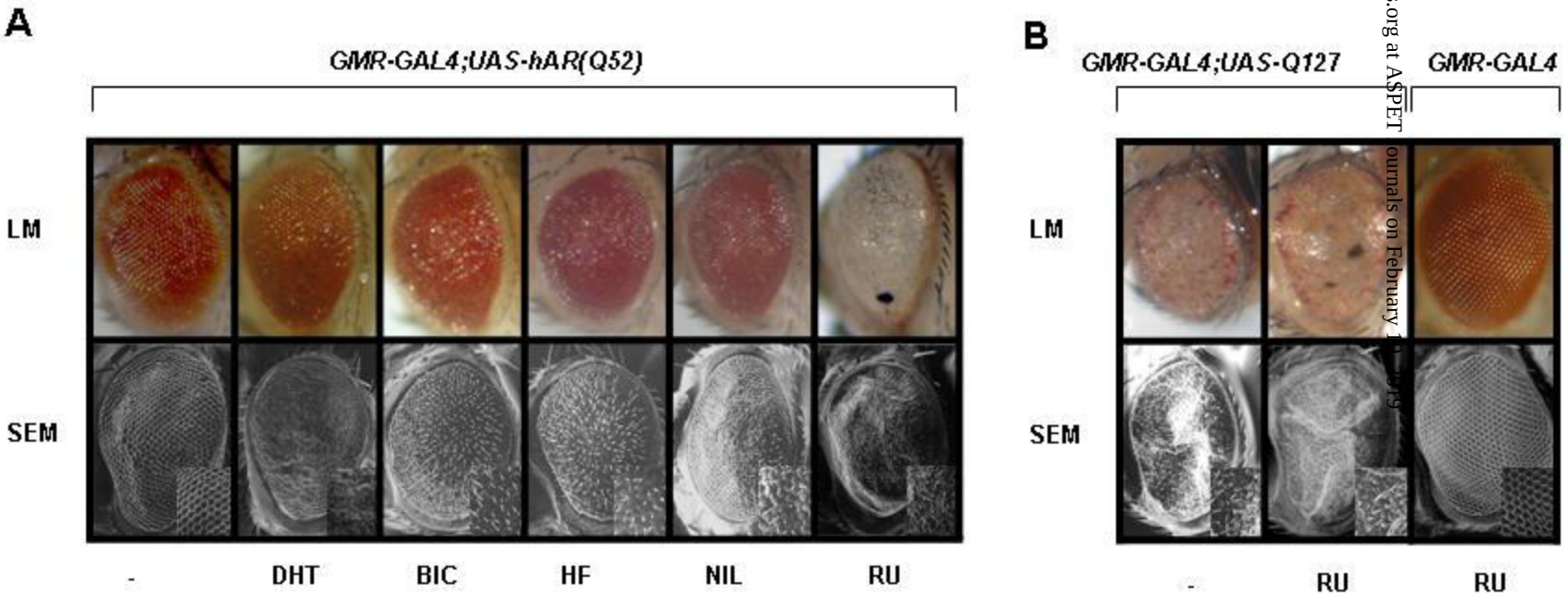

Figure 4