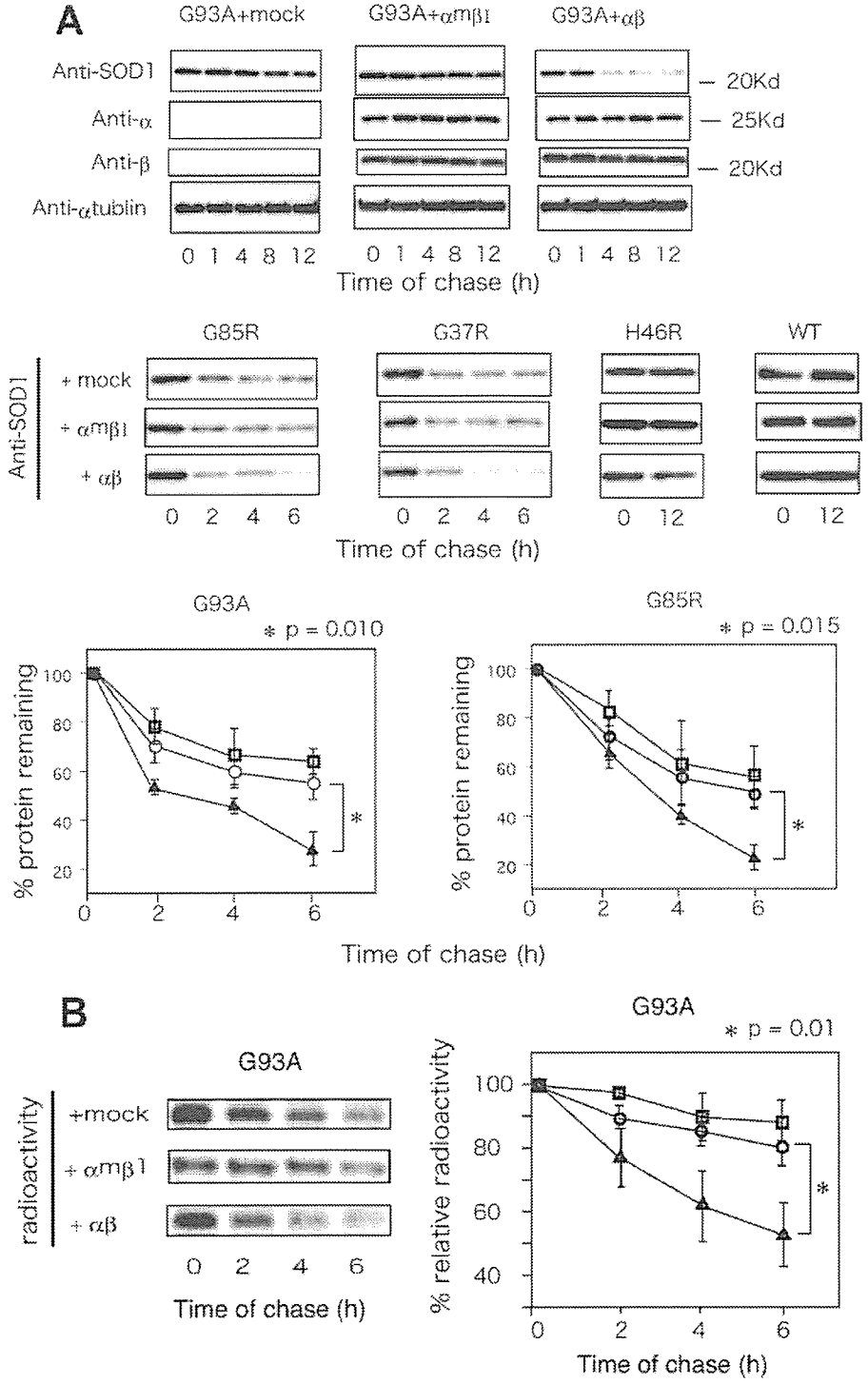

FIGURE 3. M mazei proteasome-accelerated degradation of mutant SOD1 proteins. A, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of various mutant SOD1 proteins were reduced in the presence of Mm 20 S proteasome $\alpha\beta$. The graphs represent the percentage of degraded $SOD1^{G93A}$ and $SOD1^{G85R}$ proteins in three independent experiments. The error bars indicate S.D. B, pulse-chase analysis (see "Experimental Procedures") showing that the degradation of $SOD1^{G93A}$ was accelerated in the presence of Mm 20 S proteasome $\alpha\beta$. Circle, mock; triangle, $\alpha\beta$; square, $\alpha m\beta 1$. Error bars, S.D. (n = 3).

following experiments were performed in both HEK293 and Neuro2a cells with similar results in both cell lines.

To confirm protein expression of the Mm subunits, HEK293 cells transfected with mock, α, $\Delta\alpha$, β, or $m\beta 1$ were lysed, subjected to SDS-PAGE, and immunoblotted with anti-proteasome α-subunit, anti-proteasome β-subunit, and anti-His antibodies. Fig. 1*B* demonstrates that the α- and β-subunit antibodies detected the Mm proteasome α-subunit at 26 kDa, the $\Delta\alpha$-subunit around 25 kDa, and the β-subunit at 22 kDa, respectively, and faintly recognized endogenous human proteasome subunits. A Ni^{2+}-NTA pull-down assay showed that the Mm proteasome α- and $\Delta\alpha$-subunits co-sedimented with the Mm proteasome β- and $m\beta 1$-subunits but not with mock (Fig. 1*C*), and protease activity of the pulled down samples of the cells lysed 48 h after transfection showed significantly higher chymotrypsin-like protease activity in the Mm proteasome $\alpha\beta$ than in the $\alpha m\beta 1$ or mock-transfected samples (Fig. 1*D*). This protease activity was confirmed to become gradually higher after transfection (Fig. 1*D*).

Glycerol density gradient centrifugation fractionated the $\alpha\beta$, $\Delta\alpha\beta$, and $\alpha m\beta 1$ complexes of the Mm proteasome into nearly the same fractions as those of the human 20 S proteasome subunits $\alpha 1$ and $\alpha 5$ (Fig. 1*E*, data not shown for $\Delta\alpha\beta$ and $\alpha m\beta 1$). Moreover, of the anti-His-immunoblotted bands (Fig. 1*E*), the density of staining in fractions 20–25 accounts for about 80–90% of the total anti-His staining. That these fractions constitute the majority of the anti-α staining as well suggests that about 80–90% of the β-subunit expression is incorporated into the Mm proteasome. These results suggested that the Mm proteasome α-, $\Delta\alpha$-, β-, and $m\beta 1$-subunits could properly assemble to form four stacked seven-membered rings and that an active Mm proteasome could be reproduced in mammalian cells. The cells expressing Mm proteasome $\Delta\alpha\beta$ displayed cellular toxicity, whereas the cells expressing Mm proteasome $\alpha\beta$ showed little toxicity

mally form a gated channel in the α-ring that regulates substrate entry into the 20 S proteasome (19). We also generated a mutant β-subunit with T1C ($m\beta 1$) (Fig. 1*A*). Thr-1 in the β-subunit of the archaeal proteasome is essential for proteolysis, and Thr-1 mutants lose their proteolytic activities (20). The

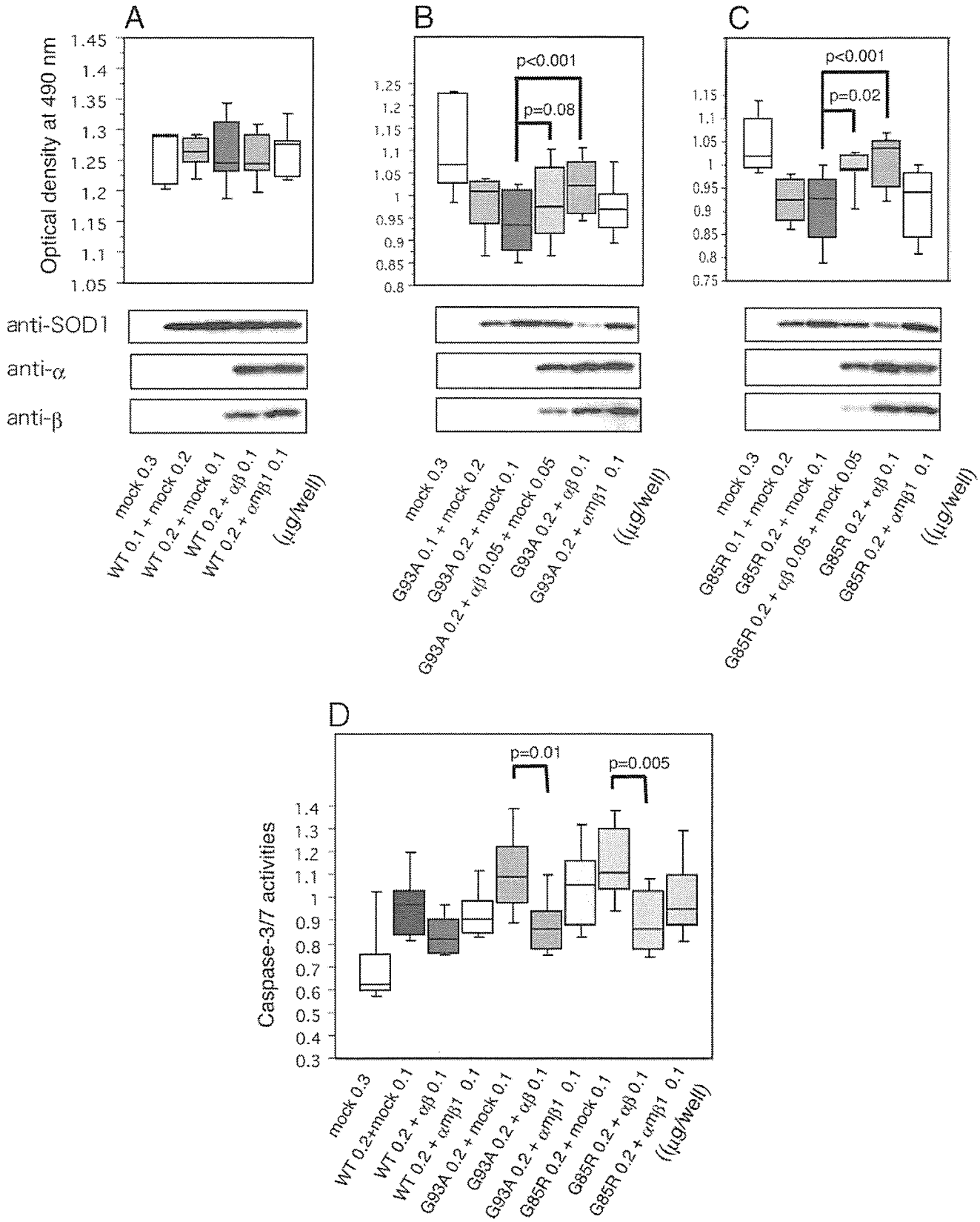

FIGURE 4. M. mazei proteasome reduces the cellular toxicity of mutant SOD1. The dose-dependent rescue effect of Mm proteasome $\alpha\beta$ expression on cell viability in SOD1^{WT}- (A), SOD1^{G93A}- (B), and SOD1^{G85R}-transfected HEK293 cells (C) as shown in MTS-based cell proliferation assays. The box plots show the median values (center line of box), the 25th (lower line of box), 75th (upper line of box), 10th (lower Tbar), and 90th (upper Tbar) percentiles in each group (n = 3 × 6 wells). The numbers indicate the dose of DNA transfected in each well of a 96-well plate ($\alpha\beta$, 0.1 μg; α, 0.05 μg; β, 0.05 μg). The expression levels of SOD1, α-subunit, and β-subunit at the analyzed points are shown. D, relative activities of cleaved caspase-3/7 were analyzed with the fluorescent caspase substrate, benzyloxycarbonyl-DEVD-R110. Production of Mm proteasome $\alpha\beta$ prevents activation of caspase-3/7. Positive control value was 3.2 ± 0.2 (S.D.) (n = 3 × 4 wells) (1 μM staurosporin, 24 h).

(data not shown); thus, further experiments were carried out with Mm proteasomes $\alpha\beta$ and $\alpha m\beta 1$.

M. mazei Proteasome Degrades Specifically Mutant Superoxide Dismutase-1—We then assessed whether the Mm proteasome actually affects mutant SOD1 protein ($SOD1^{G85R}$, $SOD1^{G37R}$, $SOD1^{G93A}$, and $SOD1^{H46R}$) expression. In cultured cells, mutant $SOD1^{G85R}$, $SOD1^{G37R}$, and $SOD1^{G93A}$ are more likely to form aggregates than is $SOD1^{H46R}$ (16), and cases of familial ALS expressing these mutant forms are also more severe than those expressing $SOD1^{H46R}$. Western blot analyses demonstrated that the levels of mutant SOD1 were markedly reduced as the expression of Mm proteasome $\alpha\beta$ increased (Fig. 2). However, wild-type SOD1 levels were not affected by the expression of Mm proteasome $\alpha\beta$. Furthermore, mutant SOD1 levels were not affected by the expression of Mm proteasome containing the $m\beta 1$-subunit in all mutant species, indicating that Mm proteasomal activity was important to reduce the levels of mutant SOD1 proteins. That the expression level of $SOD1^{H46R}$ was less affected by Mm proteasomal expression than other mutant SOD1 species may be associated with the lower toxicity of $SOD1^{H46R}$.

To determine whether the reduced levels of mutant SOD1 protein were due to accelerated degradation of mutant SOD1 or to the reduction of mutant SOD1 expression, we examined the stability of mutant SOD1 proteins expressed in Neuro2a cells co-expressed with Mm proteasome $\alpha\beta$, $\alpha m\beta 1$, or mock (Fig. 3, *A* and *B*). Chase experiments with cycloheximide, which halts all cellular protein synthesis, demonstrated mutant species-dependent acceleration in SOD1 protein degradation, whereas the expression levels of Mm proteasome α- and β-subunits did not change (Fig. 3*A*). The degree of wild-type SOD1 degradation was not affected by the expression of Mm proteasome $\alpha\beta$. Pulse-chase experiments further confirmed that ^{35}S-labeled $SOD1^{G93A}$ degradation was significantly accelerated when co-expressed with Mm proteasome $\alpha\beta$ but not with Mm proteasome $\alpha m\beta 1$ or mock (Fig. 3*B*). These facts strongly suggest that the catalytic center in the Mm proteasome β-subunit is important to accelerate the degradation of mutant SOD1 proteins.

M. mazei Proteasome Reduces Cellular Toxicities of Mutant Superoxide Dismutase-1—Next, we investigated the viability of HEK293 cells evoked by SOD1 (wild-type, $SOD1^{G93A}$, and $SOD1^{G85R}$) when co-expressed with Mm proteasome $\alpha\beta$, $\alpha m\beta 1$, or mock by the MTS-based cell proliferation assay (Fig. 4). We confirmed a linear response between cell number and optical density at 490 nm between 0.85 and 1.30 (data not shown). The viability of cells expressing wild-type SOD1 with Mm proteasome $\alpha\beta$ did not change as the transfected DNA doses of SOD1 and Mm proteasome $\alpha\beta$ increased (Fig. 4*A*). However, the viability of cells expressing mutant SOD1 was reduced as the transfected DNA dose of SOD1 increased (Fig. 4, *B* and *C*), and this reduction was prevented by the co-transfection with Mm proteasome $\alpha\beta$ but not with Mm proteasome $\alpha m\beta 1$. Toxicities of mutant SOD1 proteins are associated with the activation of caspase family proteins, especially caspase-3 (21). Using fluorescent substrates of activated caspase-3/7 as markers, we analyzed caspase-3/7 activities in the cells co-transfected with SOD1 proteins and with mock, Mm proteasome $\alpha\beta$, and $\alpha m\beta 1$. Mm proteasome $\alpha\beta$ suppressed the activation of caspase-3/7, resulting in reductions of cellular toxicities of SOD1 proteins (Fig. 4*D*). These results show that Mm proteasome $\alpha\beta$ has a protective effect against the decrease in cellular viability evoked by mutant SOD1.

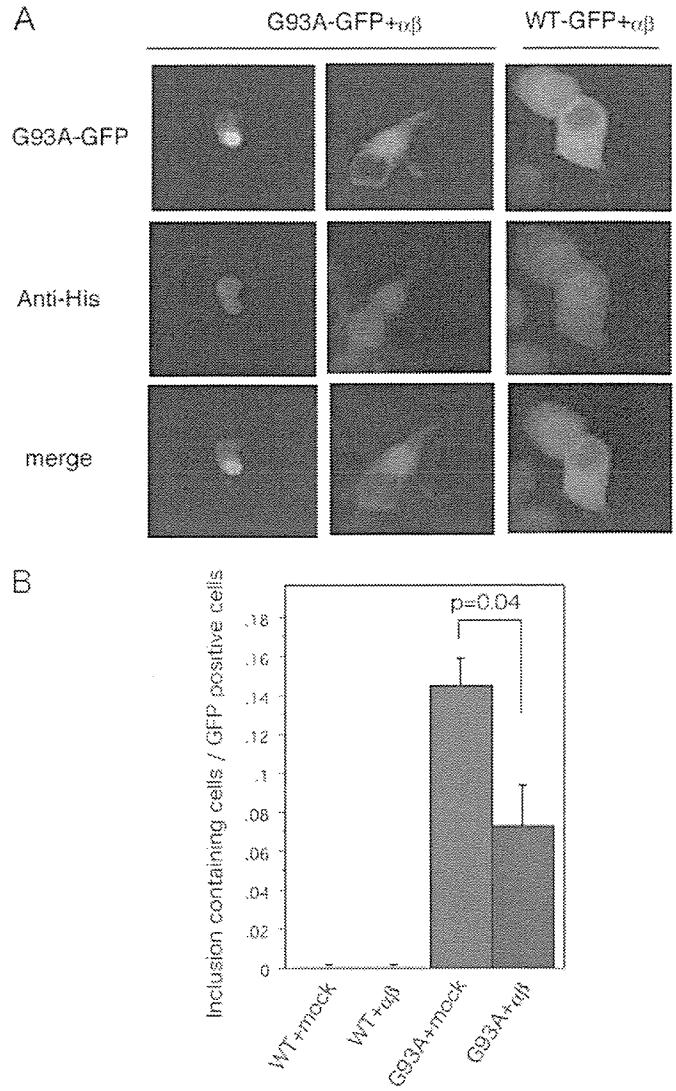

FIGURE 5. Co-localization of mutant SOD1 and M mazei proteasomes. A, Neuro2a cells grown on glass coverslips were co-transfected with $SOD1^{WT}$-GFP or $SOD1^{G93A}$-GFP and Mm proteasome α- and His-tagged β-subunit. 48 h after transfection, cells were fixed, blocked, and incubated with anti-His antibody for 24 h. After washing, samples were incubated with Alexa-546-conjugated anti-mouse antibody. $SOD1^{G93A}$ and the Mm proteasome co-localized and formed aggregates together. WT, wild-type SOD1; G93A, $SOD1^{G93A}$. B, the percentages of aggregate-positive cells among the GFP-positive cells were determined. $SOD1^{G93A}$ aggregates were significantly reduced when co-expressed with Mm proteasome $\alpha\beta$. Error bars, S.D. (n = 3). Statistical analyses were carried out by Mann-Whitney's U test.

M. mazei Proteasome Co-localizes with Aggregates Formed by Mutant SOD1—In the assembly process of the archaeal proteasome, α-subunit assembly is required for β-subunit incorporation into the proteasome (20), and since the anti-His-stained β-subunit is restricted largely to that incorporated into the Mm proteasome (Fig. 1*E*), we used anti-His staining to localize the transfected proteasome in Neuro2a cells. GFP-tagged wild-type and G93A mutant SOD1 vectors were transfected along with Mm proteasome $\alpha\beta$ into Neuro2a cells, which were then fixed and immunostained with anti-His antibody. Fig. 5*A* shows that

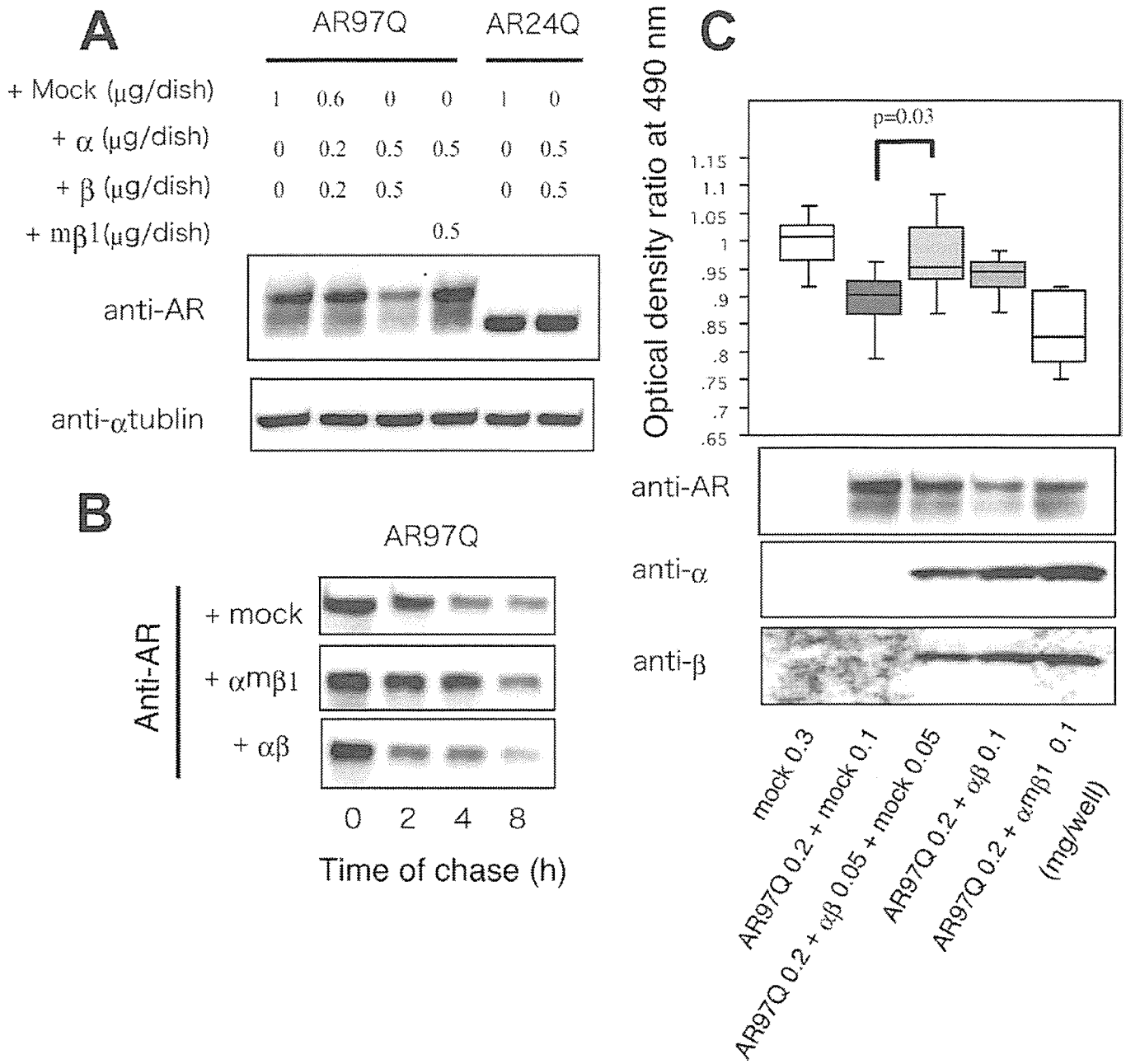

FIGURE 6. M mazei proteasome degrades mutant androgen receptor with expanded polyglutamine tract and reduces its cellular toxicity. A, Neuro2a cells grown on 6-cm dishes and co-transfected with 1 μg of AR containing either normal (24Q) or expanded (97Q) polyglutamine tract vectors and increasing doses of Mm proteasome subunits were analyzed. The levels of AR^{97Q} proteins were reduced as Mm proteasome αβ increased. B, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of AR^{97Q} proteins were decreased in the presence of Mm 20 S proteasome αβ. Transfected DNA dose/6-cm dish was as follows: AR^{97Q} (1 μg), α-subunit (0.5 μg), β-subunit (0.5 μg). C, the rescue effect of Mm proteasome αβ expression on cell viability in AR^{97Q}-transfected HEK293 cells as shown in an MTS assay. The box plots show the median values (center line of box), the 25th (lower line of box), 75th (upper line of box), 10th (lower Tbar), and 90th (upper Tbar) percentiles in each group (n = 3 × 6 wells). The numbers indicate transfected DNA dose in a well of a 96-well plate (αβ, 0.1 μg; α, 0.05 μg; β, 0.05 μg). The expression levels of AR, α-subunit, and β-subunit at analyzed points are shown.

GFP-positive $SOD1^{G93A}$ aggregates are also anti-His positive, whereas the cells expressing wild-type SOD1-GFP are diffusely stained with anti-His antibody. There were no GFP-negative inclusion bodies stained with anti-His antibody, indicating that Mm proteasome co-localizes with the inclusion bodies consisting of mutant SOD1 in the vicinity of the nucleus. The percentages of aggregate-positive cells among the GFP-positive cells were determined in Fig. 5*B*. $SOD1^{G93A}$ aggregates were significantly reduced when co-expressed with Mm proteasome αβ.

M. mazei Proteasome Degrades Specifically Mutant Androgen Receptor with Expanded Polyglutamine Tract and Reduces Its Cellular Toxicity—To demonstrate the ability of the Mm proteasome to degrade aggregation-prone proteins, we examined the AR with expanded polyglutamine tract (97-repeated glutamine; 97Q) protein, the causative protein of spinal and bulbar muscular atrophy. Similar to the results obtained with SOD1 proteins, Fig. 6*A* shows that in Neuro2a cells, the levels of mutant AR (97Q) were markedly reduced as the expression of

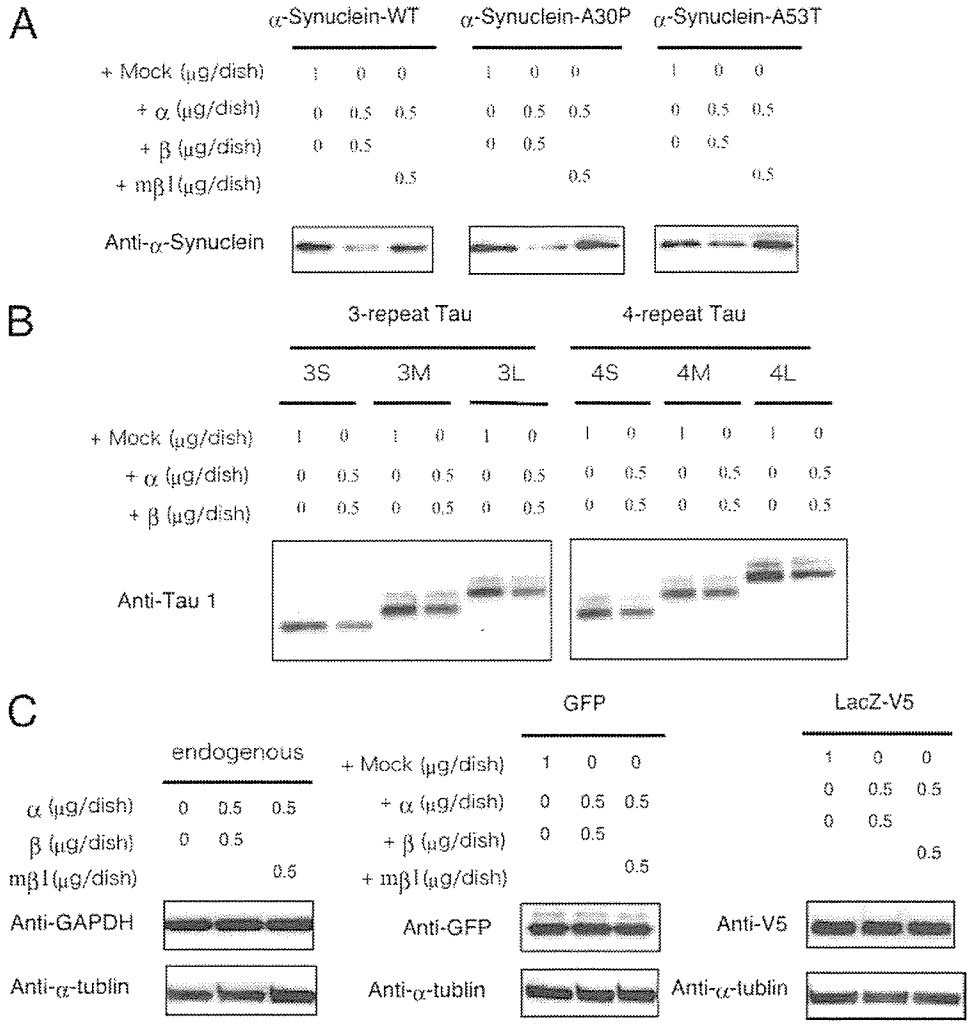

FIGURE 7. M mazei proteasome degrades aggregation-prone but not non-aggregation-prone proteins. Neuro2a cells grown on 6-cm dishes and co-transfected with Mm proteasome subunits vectors or mock and 1 μg of α-synuclein vectors (wild type, A30P, and A53T) (A), Tau vectors (six isoforms: three (3L, 3M and 3S) or four (4L, 4M and 4S) tubulin binding domains in the C-terminal portion and two (3L and 4L), one (3M and 4M), or no (3S and 4S) inserts of 29 amino acids each in the N-terminal portion) (B), or empty GFP vector or LacZ-V5 vector (C). A and B, the expression levels of all of α-synuclein and tau proteins were reduced when co-transfected with the Mm proteasome αβ. C, the expression levels of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GFP, and LacZ-V5 proteins were not changed in the presence of the Mm proteasome αβ.

Mm proteasome αβ increased, but they were unaffected by the expression of the Mm proteasome αmβ1. On the other hand, wild-type AR (24-repeated glutamine; 24Q) levels were not affected by the expression of Mm proteasome αβ. Cycloheximide-chasing analysis demonstrated that the half-life of mutant AR (97Q) was reduced in the presence of the Mm proteasome but not in the presence of the mutant Mm proteasome (Fig. 6*B*). The viability of cells expressing mutant AR (97Q) was reduced compared with wild-type AR (24Q), and this reduction was attenuated by the co-transfection with Mm proteasome αβ (Fig. 6*C*). These results show that Mm proteasome αβ can accelerate the degradation of the aggregation-prone mutant AR with expanded polyglutamine tract and possibly protect the cells from its toxicities.

M. mazei Proteasome Degrades Other Aggregation-prone Proteins but Not Non-aggregation-prone Proteins—To determine whether the Mm proteasome degrades other aggregation-prone proteins as well, we examined its effects on α-synuclein (wild-type, A53T, and A30P) and six isoforms of wild-type tau protein in Neuro2a cells. The six tau isoforms contained either three (3L, 3M, and 3S) or four (4L, 4M, and 4S) microtubule binding domains in the C-terminal portion and two (3L, 4L), one (3M, 4M), or no (3S, 4S) inserts of 29 amino acids each in the N-terminal portion. Similar to the results obtained with the mutant SOD1 and AR with an expanded polyglutamine tract, the expression levels of all α-synuclein and tau proteins were reduced in the presence of Mm proteasome αβ (Fig. 7, *A* and *B*). Although the degradations of wild-type SOD1 and AR proteins were not accelerated by Mm proteasome, the expression levels of α-synuclein including wild-type and all of the six forms of wild-type tau were reduced.

We also examined whether Mm proteasomes degrade non-aggregation-prone proteins such as GFP or LacZ. Fig. 7*C* shows that the Mm proteasome does not affect the degradation of the exogenously expressed proteins, GFP and LacZ.

DISCUSSION

In this study, we showed that the archaeal Mm proteasome α- and β-subunits properly assembled to have proteolytic activity and accelerate the degradation of aggregation-prone, neurodegeneration-associated proteins in mammalian cells. Archaeal proteasomes contain 14 identical active sites that, although originally classified as chymotrypsin-like, were later shown to cleave after acidic and basic residues (22), and they consist of only one type of each of the α- and β-subunits (6). A comparison between archaeal and eukaryotic proteasomes *in vitro* showed that archaeal proteasomes are far more active in degrading poly(Q) peptides than are eukaryotic proteasomes (9). We utilized this potential power and manageability of archaeal proteasomes to degrade abnormal proteins that could not be effectively degraded by eukaryotic proteasomes. This is the first report showing that archaeal proteasomes can work to accelerate degradation of aggregation-prone proteins in mammalian cells.

Mm proteasomes promoted degradation of mutant SOD1, AR with an expanded polyglutamine tract, wild-type and mutant α-synuclein, and six isoforms of wild-type tau. The first two proteins, mutant SOD1 and AR with an expanded polyglutamine tract, exhibit toxicity in cell culture models. Mice over-expressing these mutant proteins display abnormal aggrega-

tions in their motor neurons and significant loss of motor functions, and they have been used as disease models (23, 24). Mm proteasomes accelerated the degradation of only the mutant forms of these two proteins and not that of the nonaggregating wild-type forms. Furthermore, chasing studies (Fig. 3, *A* and *B*) confirmed our belief that Mm proteasomes directly accelerate the degradation of mutant proteins.

However, both the wild-type and two mutants of α-synuclein as well as six isoforms of wild-type tau were also degraded by Mm proteasomes (Fig. 7). α-Synuclein and tau are pathogenically different proteins from SOD1 and AR, since they are known to accumulate as wild-type proteins in the affected lesions of PD and AD, respectively. Aggregation of the presynaptic protein, α-synuclein, has been implicated in synucleinopathies, such as sporadic and familial PD, diffuse Lewy body disease, and multiple-system atrophy (25). In sporadic PD patients, wild-type α-synuclein is accumulated, and increased expression of wild-type α-synuclein is also observed (26). Proteasomal dysfunction has been thought to impair α-synuclein degradation and thereby to facilitate its aggregation (27). Three- and four-repeat wild-type tau are among the proteins characteristically detected in neurofibrillary tangles formed by paired helical filaments in sporadic AD (28). Decreased proteasomal activity has been also reported in the AD brain (29). α-Synuclein and tau are both relatively easily misfolded, which leads to the formation of aggregates, even in their wild-type forms (30, 31), thus possibly explaining why the Mm proteasomes degraded wild-type α-synuclein and tau. Mm proteasomes might be able to recognize a wide range of aggregation-prone proteins, whereas they do not affect the degradation of exogenously expressed nonaggregating proteins, such as GFP and LacZ, or abundant endogenous proteins, such as α-tubulin and glyceraldehyde-3-phosphate dehydrogenase (Fig. 7).

The question raised here is what is the molecular mechanism of such selective, mutant species-dependant degradation. Archaeal 20 S proteasomes contain proteasome-activating nucleotidase, PAN, enabling substrates to enter the proteasomes easily and effectively (8). PAN has a chaperone-like activity to unfold aggregated proteins (32) and is thought to be an evolutionary precursor to the 19 S base in eukaryotic cells (8). Archaeal recognition tags (like ubiquitin tags in eukaryotic cells) have not been identified yet. However, archaeal 20 S proteasomes have been reported to rapidly degrade polyglutamine aggregates *in vitro*, without the help of PAN (9). Here we confirmed that this PAN-independent degradation by Mm 20 S proteasomes could occur in mammalian cells. Since the pore diameter of the closed gate in 20 S proteasomes is estimated to be much smaller than that of aggregated proteins (33), the question is, how do the unfolded substrate proteins enter the 20 S proteasomes? One hypothesis might be that the α-ring in Mm proteasomes has chaperone-like activity to recognize and unfold the aggregation-prone proteins or misfolded proteins. The gated channel in the α-ring of the archaeal 20 S proteasomes is thought to regulate substrate entry into the proteasomes and is assumed to be in either an open (34) or a closed state (2, 33) *in vitro*. In our experiments, the gate-free Mm 20 S proteasome Δαβ substantially reduced cell viability, but the Mm proteasome αβ, with the "gate," had little toxic effect on the cells and, furthermore, accelerated the degradation of mutant proteins. This would be hard to explain if the gate is always in the closed state. There is a possibility that when Mm proteasomes gather, actively or passively, near aggregation-prone proteins, the α-ring opens its gate and unfolds the aggregated proteins, enabling them to enter the proteasomes to be degraded.

Some kinds of molecular chaperones, such as Hsp90, -70, and -27, have been reported to assist in the selective degradation of mutant SOD1 and AR proteins in proteasome degradation pathways (35, 17). However, neither the protein levels of molecular chaperones (Hsp90, -70, -40, and -27) nor the ubiquitylation levels of mutant SOD1 and AR were changed in the presence of Mm proteasome αβ expression (data not shown), thus supporting the idea that endogenous ubiquitin-proteasome degradation pathways possibly did not play an important role in the accelerated degradation of mutant proteins. Further study is needed to elucidate the molecular mechanisms of selective recognition of misfolded aggregation-prone proteins by Mm proteasomes.

In this paper, we demonstrated that Mm proteasomes could effectively degrade neurodegenerative disease-related aggregation-prone proteins *in vivo*. Further studies are needed to determine whether archaeal proteasomes can be available to treat diseases in which toxic gain of proteins is causative.

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REVIEW

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Modulation of Hsp90 function in neurodegenerative disorders: a molecular-targeted therapy against disease-causing protein

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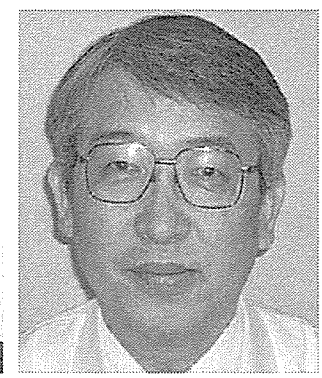
Abstract Abnormal accumulation of disease-causing protein is a commonly observed characteristic in chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and polyglutamine (polyQ) diseases. A therapeutic approach that could selectively eliminate would be a promising remedy for neurodegenerative disorders. Spinal and bulbar muscular atrophy (SBMA), one of the polyQ diseases, is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. The pathogenic gene product is polyQ-expanded androgen receptor (AR), which belongs to the heat shock protein (Hsp) 90 client protein family. 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a novel Hsp90 inhibitor, is a new derivative of geldanamycin that shares its important biological activities but shows less toxicity. 17-AAG is now in phase II clinical trials as a potential anticancer agent because of its ability to selectively degrade several oncoproteins. We have recently demonstrated the efficacy and safety of 17-AAG in a mouse model of SBMA. The administration of 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. 17-AAG accomplished the preferential reduction of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation. 17-AAG induced Hsp70 and Hsp40 in vivo as previously reported; however, its ability to induce HSPs was limited, suggesting that the HSP induction might support the degradation of mutant protein. The ability of 17-AAG to preferentially degrade mutant protein would be directly applicable to SBMA and other neurodegenerative diseases in which the disease-causing proteins also belong to the Hsp90 client protein family. Our proposed therapeutic approach, modulation of Hsp90 function by 17-AAG treatment, has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases. This review will consider our research findings and discuss the possibility of a clinical application of 17-AAG to SBMA and other neurodegenerative diseases.

Keywords Hsp90 inhibitor · Hsp90–client protein complex · Proteasomal degradation · Polyglutamine · Neurodegenerative diseases



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Introduction

Polyglutamine (polyQ) diseases are caused by the expansion of a trinucleotide CAG repeat encoding glutamine in the causative genes and, to date, nine disorders have been identified as polyQ diseases [1]. Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, was the first polyQ disease to be identified [2] and is characterized by premature muscular exhaustion, slow progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles [3]. In SBMA, the pathogenic gene product is the androgen receptor (AR), which contains an abnormally expanded polyQ. The number of polymorphic CAG repeats in the AR gene is normally 14 to 32, but it is expanded to 40 to 62 CAGs in SBMA patients [4]. A correlation exists between the number of CAG repeats and disease severity [5]. The pathologic features of SBMA are motor neuron loss in the spinal cord and brainstem [3], and diffuse nuclear accumulations and nuclear inclusions (NIs) containing the mutant AR in the residual motor neurons and certain visceral organs [6].

Heat shock protein (Hsp) 90, one of the molecular chaperones, is essential for function and stability of the AR, the C-terminus of which has a high affinity for Hsp90, inducing the conformational change required for its nuclear translocation after ligand activation [7–9]. Hsp90 functions in a multi-component complex of chaperone proteins including Hsp70, Hop (Hsp70 and Hsp90 organizing protein), Cdc37, and p23. In addition, Hsp90 is involved in the folding, activation, and assembly of several proteins, known as Hsp90 client proteins [10]. As numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded in the ubiquitin–proteasome system (UPS) by Hsp90 inhibitors, 17-allylamino-17-demethoxy-geldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now under clinical trials as a novel molecular-targeted agent for a wide range of malignancies [11]. AR also belongs to the Hsp90 client protein family and is degraded in the presence of Hsp90 inhibitors [12–14].

In view of this ability of Hsp90 inhibitors to degrade Hsp90 client proteins, we have recently demonstrated that 17-AAG markedly ameliorated polyQ-mediated motor neuron degeneration through degradation of mutant AR [15]. This is apparently different from previous strategies employed against polyQ diseases, which unavoidably allowed abnormal protein to remain and placed much value mainly on the inhibition of protein aggregation. We consider that the ability to facilitate degradation of disease-causing protein by modulation of Hsp90 function would be of value when applied to SBMA and other related neurodegenerative diseases. In this paper, we review our research findings compared with previous studies and discuss the clinical application of Hsp90 inhibitors to neurodegenerative diseases.

Development of Hsp90 inhibitors

The most classical Hsp90 inhibitor is geldanamycin (GA), a natural product that was developed as an antifungal agent [16]. Later, GA was also found to have a potent and selective anti-tumor effect against a wide range of malignancies [17]. Although GA showed potential as a novel anti-cancer agent [18], this agent was also found to have intolerable liver toxicity [19]. To overcome this GA-induced liver toxicity, scientists at the US National Cancer Institute succeeded in developing a new derivative of GA, 17-AAG, that shares its important biological activities [20] but shows less toxicity [21]. Owing to this promising derivative 17-AAG, Hsp90 inhibitors have taken a major developmental leap in their clinical applications, and 17-AAG is now in phase clinical trials with encouraging results as an anti-cancer agent [22–26]. To generate more selective and less toxic derivatives than 17-AAG, further development of Hsp90 inhibitors is also being pursued [27–29].

The anti-tumor effect of Hsp90 inhibitors was previously thought to be due to the inhibition of tyrosine kinase [30]. The mechanism subsequently proved to be based on their ability to specifically bind to the Hsp90 ATP-binding site, thereby modulating Hsp90 function [31, 32] and proteasomal degradation of Hsp90 client proteins. As numerous oncoproteins were shown to belong to the family of Hsp90 client proteins [10]. Hsp90 inhibitors are expected to become a new strategy in anti-tumor therapy [18]. Hsp90 inhibitors including GA and 17-AAG have been shown to have an advantageously higher selectivity for tumor cells compared with general anti-tumor agents [17]. Studies by Kamal et al. suggest a mechanism for this selectivity; Hsp90 in tumor cells is more likely to be incorporated in the Hsp90 multi-chaperone complex than the Hsp90 in normal cells is, thereby increasing their binding affinity to 17-AAG by more than 100-fold [33, 34].

We thought that this selectivity of Hsp90 inhibitors would also be advantageous for the treatment of neurodegenerative diseases. However, as neurodegenerative diseases generally follow a chronic progression and the medical treatment is long compared with that for malignancy, the toxic side effects of the treatments would need to be extensively suppressed. Therefore, we decided to explore the possibility of using 17-AAG as a therapeutic agent for neurodegenerative diseases by examining its effects on mutant AR in cultured cells and in a mouse model of SBMA.

A model mouse of SBMA and a potent hormonal therapy

We had previously generated transgenic mice expressing the full-length human AR containing either 24 or 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken actin promoter [35]. The mice with 97 CAG repeats (AR-97Q) exhibited progressive motor impairment, while none of those with 24 CAG repeats (AR-24Q) showed abnormal phenotypes [35]. Other laboratories have

also generated various animal models of SBMA, almost all of which display phenotypic expressions of motor dysfunction [36]. For researching a truly effective molecular-targeted therapy, it is imperative to do so in a model that approximates the native state and metabolism of the disease-causing protein in vivo. We therefore consider that our SBMA mice carrying full-length AR are more beneficial for investigating therapeutic agents than those carrying the truncated one. Our transgenic mice indeed have a very severe phenotype, which is different to some extent from the human form of the disease, but they demonstrate polyQ-induced motor neuron degeneration and provide a beneficial tool to screen therapeutic agents to rescue this condition as we previously described [37]. For further details about the clinical features and our mouse model of SBMA, please refer to Katsuno et al. [38, 39].

We have already experimentally demonstrated several therapeutic approaches using this model [35, 37, 40, 41] and consequently confirmed that leuprorelin, a lutenizing hormone-releasing hormone agonist that reduces testosterone release from the testis, significantly rescued motor dysfunction and nuclear accumulation of mutant AR in our SBMA mice. Due to its minimal invasiveness established in human and its powerful efficacy demonstrated in the above model [37], this hormonal therapy has already been in human clinical trials with encouraging results [42]. However, it is an extremely specialized therapy for SBMA and cannot be applied to other polyQ diseases [35, 37, 38]. In contrast to this hormonal therapy, 17-AAG would be a potential therapeutic agent for SBMA as well as for other related diseases [15].

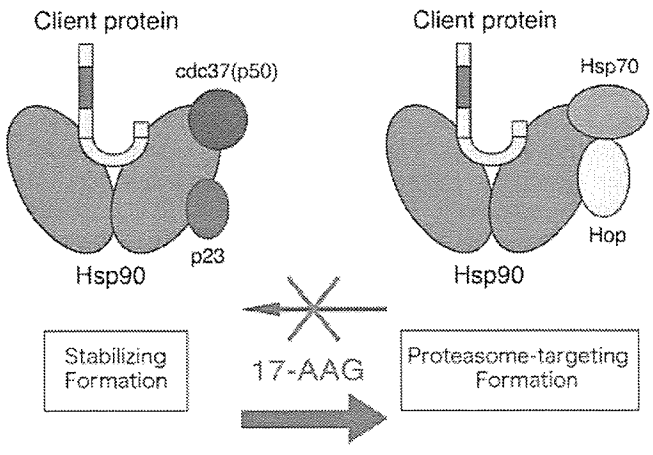

Fig. 1 Hsp90-inhibitor-induced change in Hsp90 complex. Hsp90 inhibitor (17-AAG) specifically binds ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex. To exert its effects on client proteins, Hsp90 functions in a multi-component complex of co-chaperone proteins including Hsp70, Hop, Cdc37, and p23. Two main forms of this complex exist. One complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with Cdc37 and p23. Hsp90 inhibitors block the progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form. This figure is modified from a model proposed by Neckers [14]

17-AAG alters the form of the Hsp90 complex, leading to proteasomal degradation of mutant AR

Hsp90 functions in a multi-component complex of chaperone proteins, including Hsp70, Hop, Cdc37, and p23, leading to the folding, activation, and assembly of Hsp90 client proteins [10]. In addition, the Hsp90 complexes are thought to exist in two main forms: one complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with Cdc37 and p23 [14, 43–45] (Fig. 1). Hop is known to independently bind to both Hsp90 and Hsp70, which promotes the Hsp90/Hsp70 linkage, and is thought to direct the triage decision for client proteins by bridging the Hsp90–Hsp70 interaction [45]. On the other hand, p23 is thought to modulate Hsp90 activity in the last stages of the chaperoning pathway, leading to the stabilization of Hsp90 client proteins in an ATP-dependent manner [46]. Hsp90 inhibitors, including 17-AAG, inhibit the ATP-dependent progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form, resulting in proteasomal degradation of the Hsp90 client protein [47, 48]. Steroid receptors, including the progesterone receptor and the glucocorticoid receptor, were the first Hsp90 client proteins to be identified [49, 50]. As for AR, Hsp90 is essential to maintain its high ligand-binding affinity and its stabilization [7, 12]. In practice, Hsp90 inhibitors reduce androgen ligand-binding affinity and induce the degradation of AR [12, 13].

To address the question of whether 17-AAG also promotes the degradation of the disease-causing protein of SBMA, polyQ-expanded mutant AR, we treated SH-SY5Y cells highly expressing the wild-type (AR-24Q) or mutant (AR-97Q) AR for 6 h with 36 μM 17-AAG or with dimethyl sulfoxide (DMSO) as control, in the absence or presence of the proteasome inhibitor, MG132. Immunoblot analysis demonstrated that the monomeric mutant AR decreased significantly more than the wild type did, suggesting that the mutant AR is more sensitive to 17-AAG than the wild type is. The degradation of wild-type and mutant AR by 17-AAG was completely blocked by the proteasome inhibitor, MG132 (Fig. 2a), suggesting that 17-AAG-facilitated degradation was dependent on the proteasome system as previously reported [47, 48].

Next, we examined changes in the Hsp90 chaperone complex in wild-type and mutant AR-expressing cultured cells after 17-AAG treatments. Immunoprecipitation with anti-AR antibody revealed that Hsp90-chaperone-complex-associated Hop was markedly increased, and p23 decreased in a 17-AAG dose-dependent manner, suggesting that 17-AAG resulted in the shifting of the AR-Hsp90 chaperone complex from a mature stabilizing form with p23 to a proteasome-targeting form with Hop. This chaperone complex shift appears to be very rapid as has been suggested previously [50, 51]. The loss of p23 from the mutant AR-Hsp90 complex was significantly greater than that from the wild-type one (Fig. 2b). Furthermore, these studies also strongly suggested that the mutant AR is more prone to be in the multi-chaperone complexes of

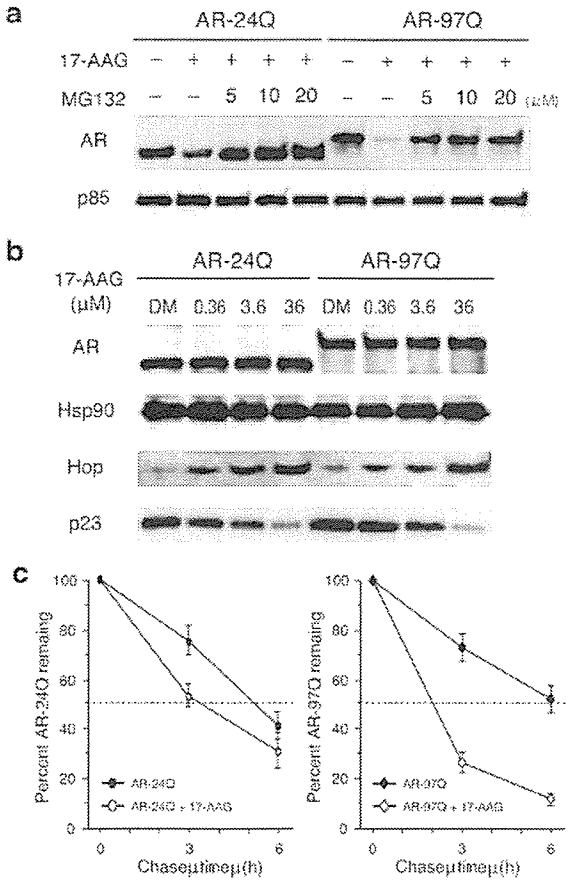

Fig. 2 a–c 17-AAG-induced changes in the AR–Hsp90 complex: correlation to proteasomal degradation. a 17-AAG treatment (36 μM, 6 h) of transfected SHSY5Y cells reduced the levels of mutant AR (AR-97Q) significantly more than the wild-type AR (AR-24Q); however, both decreases were completely blocked by the proteasomal inhibitor, MG132. b Immunoblots of lysates from transfected cells treated for 30 min with 17-AAG and immunoprecipitated with AR-specific antibody. The short time exposure to 17-AAG did not decrease the amount of mutant AR, but there were dose-dependent changes in both Hop and p23. There were no changes in the amounts of Hsp90 complexed with mutant AR. There were no changes in the expression of Hop, p23, and Hsp90 in whole lysates in the presence of 17-AAG (data not shown) c The effects of 17-AAG on the half-life of wild-type and mutant AR assessed from pulse-chase experiments. The amounts of AR-24Q remaining in the absence and presence of 17-AAG are indicated by closed circles (●) and open circles (○), respectively. The amounts of AR-97Q remaining in the absence and presence of 17-AAG are indicated by closed (♦) and open (◊) diamonds, respectively. Mutant AR was degraded more rapidly than the wild-type AR in the presence of 17-AAG. Values are expressed as means±SE (n=4)

Hsp90 with p23, which eventually enhances 17-AAG-dependent proteasomal degradation of mutant AR.

To determine whether the decrease in AR was due to protein degradation or to changes in RNA expression, the turnover of wild-type and mutant AR were then assessed with a pulse-chase labeling assay. In the presence of 17-AAG, the mutant AR and the wild-type AR had half-lives of 2 h and 3.5 h, respectively (Fig. 2c), while the mRNA levels for both the wild-type and mutant AR were quite similar [15]. These data indicate that 17-AAG preferentially degrades the mutant AR protein without altering mRNA levels. These in vitro studies indicated that the mutant AR was a good target protein of 17-AAG. To determine if it would also be preferentially degraded in vivo, we next examined the effects of 17-AAG in SBMA transgenic mice.

17-AAG ameliorates the phenotype in a mouse model of SBMA mouse without detectable toxicity

We administrated 17-AAG at doses of 2.5 or 25 mg/kg to males of the transgenic mouse model carrying full-length human AR with either 24Q or 97Q. The disease progression of AR-97Q mice treated with 25 mg/kg 17-AAG (Tg-25) was significantly ameliorated, and that of mice treated with the 2.5 mg/kg 17-AAG (Tg-2.5) was also mildly ameliorated (Fig. 3a). The AR-97Q mice treated with vehicle only (Tg-0) showed motor impairment assessed by the Rotarod task as early as 9 weeks after birth while the Tg-25 mice showed initial impairment only 18 weeks after birth and with less deterioration than the Tg-0 mice ($P<0.005$; Fig. 3a). Tg-2.5 mice showed intermediate levels of impairment in Rotarod performance (Fig. 3a). 17-AAG also significantly prolonged the survival rate of the Tg-2.5 and Tg-25 mice compared with the Tg-0 mice ($P=0.004$ and $P<0.001$, respectively; Fig. 3a). No lines were distinguishable in terms of body weight at birth; however, by 16 weeks, the Tg-0 mice showed obvious differences in body size, muscular atrophy, and kyphosis compared with the Tg-25 mice (Fig. 3b).

When mouse tissues were immunohistochemically stained for mutant AR using the 1C2 antibody, which specifically recognizes expanded polyQ, quantitative analysis revealed marked reductions in 1C2-positive nuclear accumulation in the spinal motor neurons and muscles of the Tg-25 mice compared with those of the Tg-0 mice (Fig. 3c).

Western blot analysis from lysates of the spinal cord and muscle of AR-97Q mice revealed high molecular weight mutant AR protein complex retained in the stacking gel as well as a band of monomeric mutant AR, whereas only the band of wild-type monomeric AR was visible in tissue from the AR-24Q mice (Fig. 3d). 17-AAG treatments significantly diminished both the high molecular weight complex and the monomer of mutant AR in the spinal cord and muscle of the AR-97Q mice but only slightly diminished the wild-type monomeric AR in AR-24Q mice (Fig. 3d). 17-AAG treatments decreased the amount of monomeric AR in AR-97Q mice by 64.4% in the spinal cord and 45.0% in the skeletal muscle, but only 25.9 and 12.5%, respectively, in AR-24Q mice (Fig. 3d). Thus, the reduction rate of the monomeric mutant AR was significantly higher than that of the wild-type AR in both spinal cord and skeletal muscle ($P<0.001$ and $P<0.01$ respectively). The levels of wild-type and mutant AR mRNA were similar in the respective mice treated with 17-AAG [15]. These observations indicate that 17-AAG markedly reduces not only the high molecular weight

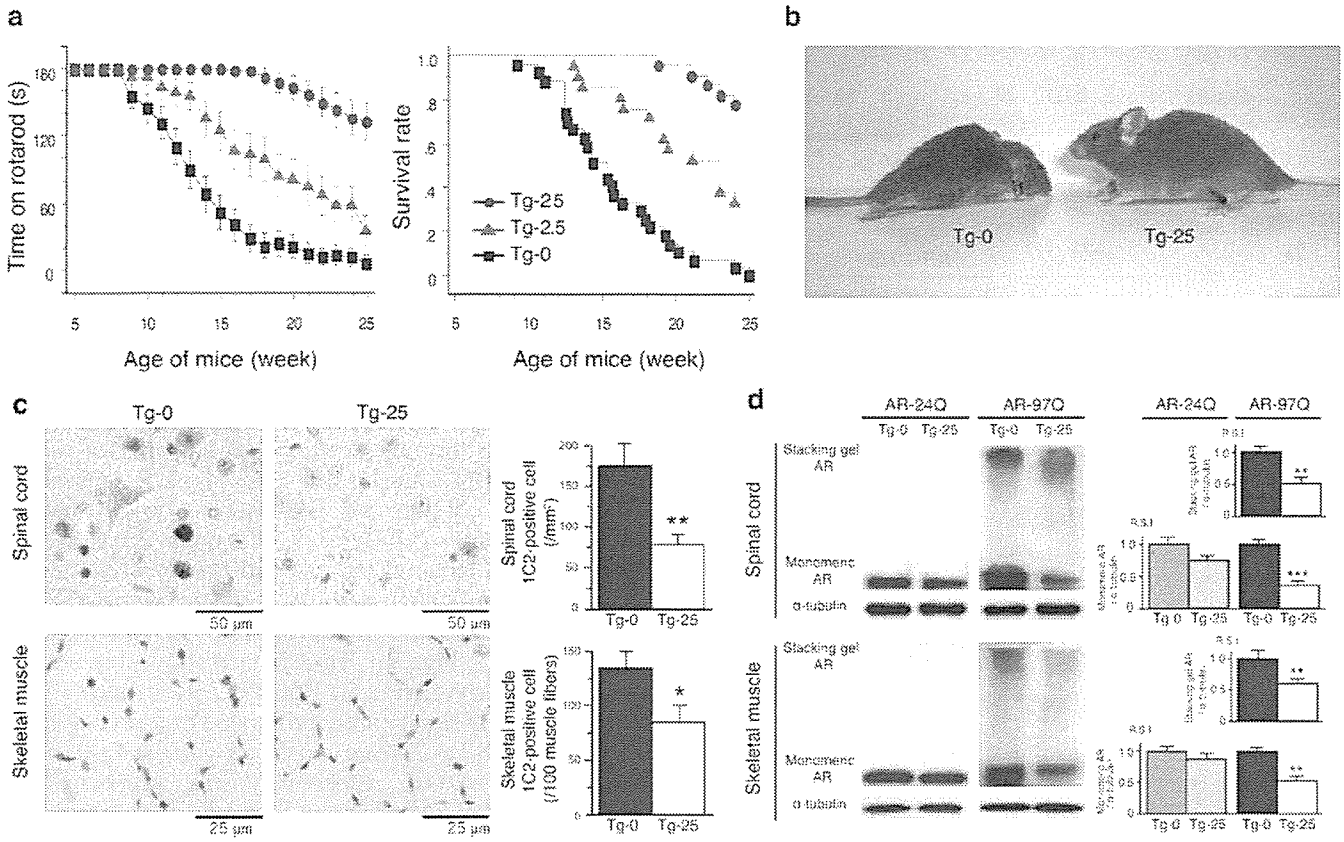

Fig. 3 a–d Effects of 17-AAG on transgenic SBMA mice. a Tg-0, Tg-2.5, and Tg-25 represent AR-97Q mice treated with vehicle alone and 2.5 and 25 mg/kg 17-AAG, respectively (each group, n= 27). The Tg-25 remained longer on the Rotarod than the Tg-0 mice did. A Kaplan–Meier plot shows the prolonged survival of Tg-2.5 and Tg-25 mice compared with the Tg-0 mice, which were all dead by 25 weeks of age (P=0.004, P<0.001, respectively). 17-AAG was less effective at the dose of 2.5 than 25 mg/kg in all parameters tested. b Representative photographs of a 16-week-old Tg-0 mouse (left) reveal an obvious difference in size and illustrate muscular atrophy and kyphosis compared with an age-matched Tg-25 mouse (right). c. Immunohistochemical staining with 1C2 antibody showed marked differences in diffuse nuclear staining and nuclear inclusions between DMSO-treated mice (Tg-0) and 17-AAG-treated (Tg-25) mice in the spinal anterior horn and skeletal muscle, respectively. There was a significant reduction in 1C2-positive cell staining in the spinal cord (**P<0.01) and skeletal muscle (*P<0.05) in the Tg-25 compared with the Tg-0. Values are expressed as means±SE (n=6). d Western blot analysis of tissue from AR-24Q and AR-97Q mice probed with an AR-specific antibody. In both spinal cord and muscle of mice treated with 17-AAG, there was a significant decrease in the amount of complexed, mutant AR in the stacking gel and monomeric mutant AR in AR-97Q mice, but only slightly less monomeric wild-type AR in AR-24Q mice compared with that from their respective, untreated control mice. Results of a densitometric analysis demonstrated that the 17-AAG-induced reduction of monomeric mutant AR was significantly greater than that of the wild-type monomeric AR. 17-AAG resulted in a 64.4% decline in monomeric mutant AR in the spinal cord and a 45.0% decline in the skeletal muscle, whereas, there was only a 25.9% decline in the spinal cord and a 12.5% decline in the skeletal muscle of AR-24Q mice. These results show significant differences in the reduction rate between wild-type and mutant AR in both spinal cord and skeletal muscle. Values are expressed as means±SE (n=5). Statistical differences are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001)

mutant AR complex but also the monomeric mutant AR protein by preferential degradation of mutant AR.

17-AAG facilitates the degradation of monomeric mutant AR, reducing its aggregation; a therapeutic approach that directly targets the disease-causing protein

In both cultured cells and transgenic SBMA mice, we have demonstrated both the efficacy and safety of 17-AAG [15]. Among the other proposed therapeutic approaches we have previously examined [35, 37, 40, 41], the efficacy of 17-AAG most closely approximated the very successful hormonal therapy using the LH-RH analog, leuprolein [15]. But, unlike leuprorelin, the Hsp90 inhibitor 17-AAG holds enormous potential for application to a wide-range of neurodegenerative diseases in addition to SBMA as previously reported [52–54]. For development of Hsp90 inhibitor treatment in neurological disorders, we regard this general versatility as very important.

In neurodegenerative diseases, recent studies have shown that disease-causing proteins in the process of aggregating have more toxic consequences than they do in either the nascent state or when in NI [55]. NIs have been thought to be a beneficial coping response to toxic mutant protein [56]. We have accumulated several pieces of data demonstrating that 17-AAG is capable of reducing aggregated protein in animal models of SBMA [15]. In both Western blot and filter trap analyses in AR-97Q

models, 17-AAG significantly diminished the insoluble high molecular weight complex of mutant AR as well as the soluble monomer. Moreover, in an immunostaining study of nervous tissue in AR-97Q mice, 17-AAG also significantly reduced diffuse nuclear staining. In SBMA patients, the extent of diffuse nuclear accumulation of mutant AR in motor and sensory neurons of the spinal cord was closely related to CAG repeat length [6]. We consider that 17-AAG had a curative effect on SBMA mice by reducing these toxic proteins as well as the soluble monomeric form.

It is difficult to determine whether 17-AGG facilitates the degradation of, specifically, these toxic intermediate proteins, as 17-AAG has the potent ability to also degrade their precursors (i.e., the monomers). One possible mode of 17-AAG action is that it may inhibit the aggregation of mutant AR via Hsp70 and Hsp40 induction. The pharmacological induction of Hsp70 and Hsp40 using Hsp90 inhibitors has already been shown to inhibit polyQ-induced abnormal aggregation of the huntingtin protein [57]. However, as 17-AAG displayed only a limited ability to induce Hsp70 and Hsp40 in mouse tissue [15], we think that the large decrease in AR seen in the insoluble fraction in vivo, rather than being a result of HSP induction, may be due to 17-AAG's potent ability to degrade the soluble monomeric form of mutant protein, thereby preventing aggregation in the first place. There is no doubt that a reduction of the main culprit protein must have curative properties against various neurodegenerative diseases. In fact, one therapeutic approach that directly reduced abnormal protein using RNA interference has already proved beneficial in various mouse models of polyQ diseases and amyotrophic lateral sclerosis [58–60].

Hsp70 is also known to accelerate proteasome-dependent degradation of polyQ abnormal protein [40, 61]. However, in our hands, mutant AR was markedly decreased after 17-AAG treatment even when Hsp70 and Hsp40 induction was completely blocked in the presence of a protein synthesis inhibitor [15], strongly suggesting that 17-AAG contributes to the preferential degradation of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation rather than via Hsp70 and Hsp40 induction. Therefore, we think that, to reap the most therapeutic benefits, Hsp90 inhibitors should be applied against neurodegenerative diseases in which the causative protein is, like AR, an Hsp90 client protein.

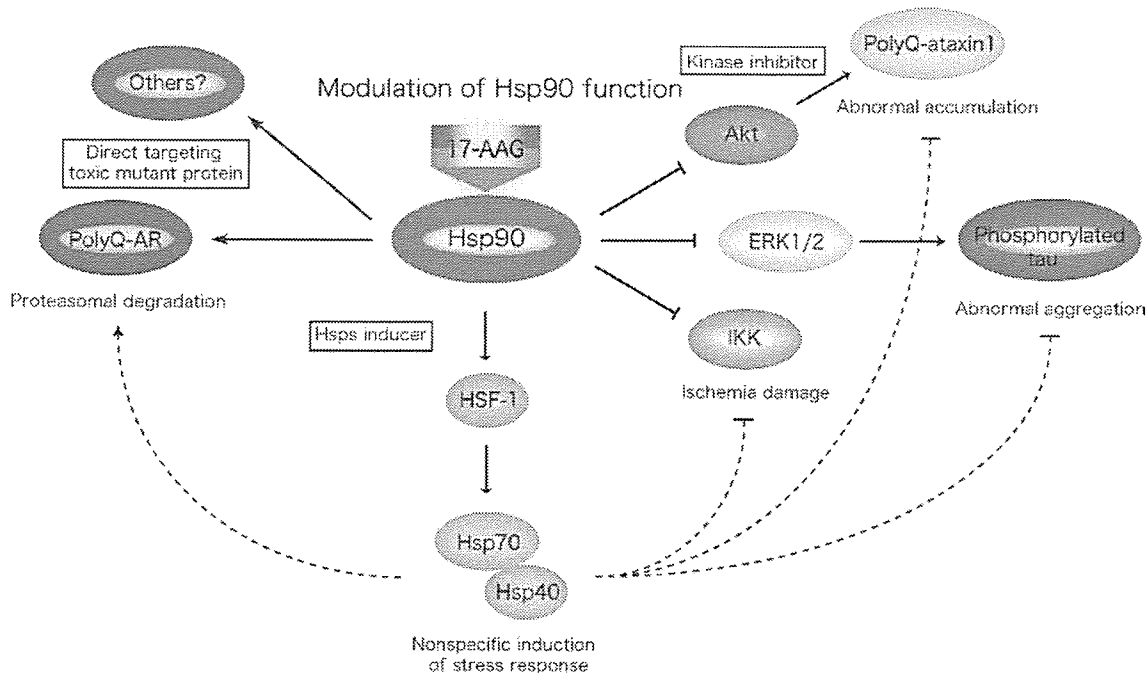

Fig. 4 Clinical application of Hsp90 inhibitors in neurological disorders. 17-AAG specifically binds to the Hsp90 ATP-binding site and disrupts its ATP-dependent function, resulting in inactivation and degradation of Hsp90 client proteins. Therapeutic approaches using Hsp90 inhibitors have now emerged that are unrelated to their role in inducing HSPs. In SBMA, where it may have its most effective potential, 17-AAG directly accelerates proteasomal degradation of the disease-causing protein, polyQ-expanded AR. However, it is still unclear whether other neurodegenerative disease-causing proteins are also Hsp90 client proteins. Many kinases involved in signal transduction do belong to the family of Hsp90 client proteins targeted by 17-AAG. ERK is associated with stabilizing phosphorylated tau. 17-AAG reduces the total amount of phosphorylated tau and its abnormal aggregation by inhibiting ERK activation. Following this same mechanism, 17-AAG might also reduce the abnormal accumulation of ataxin-1 by inhibiting Akt activation or alleviate cerebral infarction by inhibiting IKK activation, which is also an Hsp90 client protein, as well as by inducing HSPs. Hsp90 inhibitors are known to nonspecifically induce HSPs, although this effect was quite limited in our mouse model of SBMA. The induction of HSPs by Hsp90 inhibitors seems to play a supplementary role in neurodegenerative disorders

17-AAG functions with preserved UPS function

As we demonstrated in an in vitro study, 17-AAG-induced degradation requires a well-preserved proteasome function [15]. However, a question concerning the usefulness of this pharmacological approach to facilitate a self-clearing system has been raised [62]. It is generally accepted that the ubiquitin–proteasome system (UPS) is strongly involved in the pathology of polyQ diseases, as many components of the ubiquitin–proteasome and molecular chaperones are known to co-localize with polyQ-containing NIs [63, 64]. Previous reports of studies performed in cultured cell models suggested that an impairment of the UPS is probably a common pathology in polyQ diseases [65–67]. If this hypothesis were true, 17-AAG could not exert its pharmacological effect on polyQ diseases; its therapeutic effects are dependent on a preserved proteasome function [15, 47, 48, 62].

In this regard, recent studies using in vivo proteasome assays have raised serious questions concerning the impaired UPS hypothesis of polyQ diseases [68–70]. It has been reported that neuronal dysfunction developed without significant impairment of the UPS in a mouse model of SCA7 [69]. Consistent with this, it was also demonstrated that proteasome impairment did not contribute to pathogenesis in a mouse model of Huntington's disease (HD) [70]. Furthermore, in conditional mouse models of polyQ disease, genetic loss of the abnormal gene product led to a rapid clearance of pre-existing polyQ-mediated NIs and reversible improvement of the abnormal phenotypes [71, 72]. If the UPS were irreversibly damaged in polyQ diseases, then pre-existing NIs could not be diminished.

While it remains unclear what the difference is in proteasome function in in vitro and in vivo models, our research in a mouse model of SBMA indicates that impairment of the UPS is not a major etiology, at least in in vivo models of polyQ diseases. We therefore consider that treatment with 17-AAG, which takes advantage of a self-clearing system to target disease-causing proteins, is a reasonable therapeutic strategy against polyQ-related and other neurodegenerative diseases.

The expected beneficial effects of 17-AAG against other neurodegenerative diseases

Among neurodegenerative-disease-causing proteins, only AR in SBMA is established as an Hsp90 client protein at this time. It will be interesting to assess whether other neurodegenerative-disease-causing proteins also belong to the family of Hsp90 client proteins. Recent studies have already revealed that some Hsp90 client proteins exerted adverse influences on several neurological disorders [73–75], indicating that the clinical application of Hsp90 inhibitors could expand beyond the treatment of oncological diseases. With reference to previous reports, we now discuss the possibility that 17-AAG could be applicable to neurodegeneration other than SBMA (Fig. 4).

Using 17-AAG as an inducer of HSPs

Hsp90 inhibitors are known to possess the unique pharmacological effect of inducing a stress response and, in addition to their use as anti-cancer agents, have also been developed as pharmacological HSP inducers [52, 76]. This pharmacological effect has already been confirmed in human clinical trials [22]. Murakami et al. were the first to show that the Hsp90 inhibitor herbimycin had the ability to induce Hsp70 in various cultured cell models [77]. Thereafter, Hsp90 proved to be a repressor of heat transcription factor (HSF-1) [78]. Hsp90 inhibitors cause a disassociation of HSF-1 from the Hsp90 complex and a trimerization of HSF-1, thereby resulting in HSP activation. Based on the ability to induce HSPs, Hsp90 inhibitors have also been applied to non-oncological diseases [52].

A great number of reports revealed that forced overexpression of Hsp70 resulted in acquisition of tolerance against various types of stresses and protection against apoptosis in various disease models [79]. In a wide range of polyQ disease models, both genetic and pharmacological overexpression of HSPs has been shown to suppress aggregate formation and cellular toxicity [63, 80, 81]. There is no doubt that HSP induction is beneficial for various neurodegenerative diseases [54]. We have also demonstrated that both genetic and pharmacological overexpression of Hsp70 significantly ameliorated expression of the abnormal phenotype in a transgenic mouse model of SBMA [40, 82].

Taking advantage of HSP induction, many studies have already showed that Hsp90 inhibitors exerted potential neuroprotective effects in: a model of HD [57, 83, 84], tauopathies [28, 85–87], Parkinson's disease [88–90], stroke [91, 92], and autoimmune encephalomyelitis [93]. In addition, Hsp90 inhibitors themselves have been shown to have some neuroprotective effects against various stresses, such as drug-induced toxicity, oxidative stress, and oxygen-glucose deprivation [94–97]. As for polyQ diseases, Sittler et al. [57] first showed that GA significantly suppressed aggregation of mutant huntingtin in a cultured cell model of HD via induction of the Hsp70 and Hsp40 heat shock response. Thus, the enhancement of cellular defenses using Hsp90 inhibitors is a very reasonable clinical application for neurodegenerative diseases.

In polyQ diseases, Bates and his colleagues [83] showed a progressive decrease in the expression of Hsp70 and Hsp40 in a mouse model of HD, which was also observed in our SBMA model [82]. The ability of Hsp90 inhibitors to significantly induce HSPs has been demonstrated only in cultured cell models and in the fly model, but not in mammals. Therefore, further investigation should be performed to address how much Hsp90 inhibitors can induce HSPs in mouse models of neurodegenerative disorders other than SBMA. Although it appears to be obvious that it would be advantageous for the treatment of neurodegeneration, in inducing HSPs by Hsp90 inhibitors, in view of our research finding in in vivo models, it would be unadvisable to rely only on the induction of nonspecific HSPs for human clinical trials.

Using 17-AAG as a kinase inhibitor in neurodegeneration

Phosphorylated tau is a representative disease-causing protein associated with tauopathies including fronto-temporal dementia, progressive supranuclear palsy, corticobasal degeneration, and multiple system atrophy. It is interesting to note that phosphorylated tau is a targeted protein of Hsp90 inhibitors [28, 85, 86]. Dou et al. recently showed that GA and 17-AAG indirectly blocked abnormal tau phosphorylation by inhibition of the Raf–MEK–extracellular signal-regulated kinase (ERK) pathway [98], of which upstream kinase Raf is also an Hsp90 client protein [10, 99]. ERK is known to mediate the activation and stabilization of phosphorylated tau [100, 101]. Along these same lines, LaFevre-Bernt and Ellerby [102] demonstrated that polyQ-expanded, mutant-AR-mediated neuronal cell death by ERK activation and that selective inhibition of the ERK pathway reduced polyQ-induced cell death. Based on this mechanism of inhibiting ERK activation, 17-AAG might also ameliorate abnormal phenotypic expression in the mouse model of SBMA. Furthermore, in other neurodegenerative disorders, evidence has accumulated suggesting that ERK activation is an important executor of neuronal damage [103–106]. Hsp90 inhibitors are well known to have the ability to inhibit various kinase activity [10]. This pharmacological effect of Hsp90 inhibitors, to reduce abnormal kinase activity, could be applied to neurodegenerative diseases as well as oncological diseases and could have far-reaching influence on the clinical application of Hsp90 inhibitors.

Zoghbi and colleagues demonstrated that Akt/PI3K was essential for stabilization and accumulation of mutant ataxin-1 in the polyQ-associated disease SCA1 [73, 107]. Akt is also an Hsp90 client protein, whose activity is significantly reduced by Hsp90 inhibition [108, 109]. Thus, reduction of Akt kinases activity by Hsp90 inhibition might be a therapeutic approach for SCA1. Although Akt/PI3K is believed to be a major pathway mediating survival signals in neuronal cells [110], their finding raises an issue about this hypothesis.

Hsp90 inhibitors have been found to have some neuroprotective effects such as on axonal regeneration in cultured cell models [94, 111]. Koprivica et al. demonstrated that epidermal growth factor receptor (EGFR) activation mediates inhibition of axon regeneration [74]. That EGFR is also an Hsp90 client protein [112, 113] might help explain how Hsp90 inhibition is related to axonal regeneration. In another example, GA markedly attenuates ischemic brain damage [91, 92] and IκB kinase (IKK), an Hsp90 client protein [114], plays an important role in ischemia-induced neuronal death [75, 115], suggesting that GA may ameliorate ischemia brain damage by reducing IKK activity as well as by inducing HSPs.

There is a caveat to this suggestion, however. If 17-AAG is to be applied clinically to treat neurodegenerative diseases with the expectation of reducing abnormal kinase activity, we should also keep in mind the possibility that 17-AAG might also inhibit some kinase activation that exerts cytoprotective effects against neuron degeneration. Taking HD as an example, the efficacy of GA has already been shown based on its ability to induce HSPs [57, 83]. However, a report recently released by Apostol et al. showed that ERK1/2 activation protects against mutant huntingtin-induced toxicity [116]. Furthermore, in a cultured cell model of HD carrying full-length huntingtin, various kinase activities were inhibited by mutant polyQ-expanded huntingtin, but not by normal huntingtin [117, 118]. If ERK activation plays a major role in protecting against HD phenotype expression, there is concern that 17-AAG might exert an adverse affect on HD by inhibiting ERK activation. Before applying 17-AAG to a neurological disorder, we should assess whether the kinase targeted by Hsp90 inhibitor is a true exacerbating factor for the pathology. While there may be some debate over whether 17-AAG should be used as a kinase inhibitor in neurodegeneration, if the application of 17-AAG is suitably performed, this agent would be expected to effectively inhibit abnormal kinase activity in several neurological disorders, possibly leading to cures for these diseases. Hence, this strategy is also of value to extend the versatility of Hsp90 inhibitors as therapeutic agents for neurological disorders.

Conclusion

When considering a role for molecular chaperones in neurological disorders, it should be said that Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases, as these chaperones have the desirable ability to refold abnormal proteins or to carry them to degradation as a part of the system of protein quality control [54, 119]. Compared with this, Hsp90 is not known to directly fold non-native proteins but rather to bind to substrate proteins only at a late stage of folding [120]. Considering our research findings and those of the other above-mentioned reports, in addition to its role in malignancies, Hsp90 exerts an adverse influence on the nervous system in some situations. In this case, it is reasonable to consider modulating Hsp90 function appropriately.

The ability of 17-AAG to facilitate the degradation of abnormal toxic protein through the modulation of Hsp90 function would be directly applicable to SBMA and probably other neurodegenerative disorders as well. But we should keep in mind that 17-AAG is not a panacea for neurological disorders because it has only limited ability to induce Hsp70 and Hsp40 in vivo. 17-AAG is expected to exert the most effective therapeutic potential against diseases in which the main etiological factor is mediated by Hsp90 client proteins, like AR in SBMA. We believe that more research should be invested in examining the effects of Hsp90 inhibitors on neurodegeneration and that suitably modulating Hsp90 function has great potential to become a new molecular-targeted therapy against a wide range of neurodegenerative diseases.

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Natural history of spinal and bulbar muscular atrophy (SBMA): a study of 223 Japanese patients

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Spinal and bulbar muscular atrophy (SBMA) is an adult-onset motoneuron disease caused by a CAG-repeat expansion in the androgen receptor (AR) gene and for which no curative therapy exists. However, since recent research may provide opportunities for medical treatment, information concerning the natural history of SBMA would be beneficial in planning future clinical trials. We investigated the natural course of SBMA as assessed by nine activities of daily living (ADL) milestones in 223 Japanese SBMA patients (mean age at data collection = 55.2 years; range = 30–87 years) followed from 1 to 20 years. All the patients were diagnosed by genetic analysis. Hand tremor was an early event that was noticed at a median age of 33 years. Muscular weakness occurred predominantly in the lower limbs, and was noticed at a median age of 44 years, followed by the requirement of a handrail to ascend stairs at 49, dysarthria at 50, dysphagia at 54, use of a cane at 59 and a wheelchair at 61 years. Twenty-one of the patients developed pneumonia at a median age of 62 and 15 of them died at a median age of 65 years. The most common cause of death in these cases was pneumonia and respiratory failure. The ages at onset of each ADL milestone were strongly correlated with the length of CAG repeats in the AR gene. However CAG-repeat length did not correlate with the time intervals between each ADL milestone, suggesting that although the onset age of each ADL milestone depends on the CAG-repeat length in the AR gene, the rate of disease progression does not. The levels of serum testosterone, an important triggering factor for polyglutamine-mediated motoneuron degeneration, were maintained at relatively high levels even at advanced ages. These results provide beneficial information for future clinical therapeutic trials, although further detailed prospective studies are also needed.

Keywords: natural history; motoneuron disease; SBMA; Kennedy disease; ADL milestone

Abbreviations: ADL = activities of daily living; ALT = alanine aminotransferase; AR = androgen receptor; AST = aspartate aminotransferase; CK = creatine kinase; HbA1c = haemoglobin A1c; SBMA = spinal and bulbar muscular atrophy

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Introduction

Spinal and bulbar muscular atrophy (SBMA) is a neurodegenerative disorder of motoneurons characterized by proximal limb muscular atrophy, bulbar involvement, marked contraction fasciculation, hand tremor and gynaecomastia (Kennedy et al., 1968; Sobue et al., 1989). SBMA is caused by a CAG-repeat expansion in the first exon of the androgen receptor (AR) gene on the X-chromosome (La Spada et al., 1991). Similar to other triplet repeat diseases, the age at onset of disease has been inversely linked to the size of the CAG-repeat expansions (Andrew et al., 1993; Sasaki et al., 1996; Rosenblatt et al., 2003). For example, an association between the age at onset of limb muscle weakness and the CAG-repeat length has been demonstrated (Doyu et al., 1992; Igarashi et al., 1992; La Spada et al., 1992; Shimada et al., 1995; Sinnreich et al., 2004). Nuclear accumulation of mutant AR with expanded polyglutamines in motoneurons, as well as in other cells, has been shown to be a major pathogenic process (Li et al., 1998a, b; Adachi et al., 2005). However, the progression and prognosis of SBMA has not been assessed in detail, particularly concerning the influence of CAG-repeat size, the decline of the activities of daily living (ADL) with disease progression and the determination of functional