cells, using [^{35}S] labeled Met/Cys. The protein levels of SOD1^{G85R} and SOD1^{G93A} declined more rapidly with Dorfin coexpression. Dorfin-CHIP^{L} remarkably declined in both SOD1^{G85R} and SOD1^{G93A} (Figs. 6A, C). Dorfin and Dorfin-CHIP^{L} had similar expression levels at 0 h of this experiment (Fig. 6E). As compared to Mock, Dorfin showed significant declines of both SOD1^{G85R} at 3 h ($p<0.001$) and 6 h ($p<0.05$) after labeling, as shown in a previous study (Niwa et al., 2002). Dorfin-CHIP^{L} also significantly accelerated the decline of SOD1^{G85R} at 3 h ($p<0.001$) and 6 h ($p<0.05$) after labeling again as compared to Mock. At 3 h after labeling, a significant difference between Dorfin-CHIP^{L} and Dorfin was present with respect to SOD1^{G85R} degradation ($p<0.05$). As compared to Dorfin, Dorfin-CHIP^{L} also tended toward accelerated SOD1^{G85R} degradation at 6 h after labeling (Fig. 6B). Similarly, Dorfin showed significant declines of SOD1^{G93A} at 3 h ($p<0.05$) and 6 h ($p<0.05$) after labeling, and Dorfin-CHIP^{L} significantly accelerated the declines of SOD1^{G93A} at 3 h ($p<0.01$) and 6 h ($p<0.01$) after labeling as compared to Mock. A significant difference between Dorfin-CHIP^{L} and Dorfin was present at 6 h in SOD1^{G93A} degradation ($p<0.05$) (Fig. 6D).

Attenuation of the toxicity of mutant SOD1 and decrease in the formation of visible aggregations of mutant SOD1 in cultured neuronal culture cells

The ability of Dorfin-CHIP chimeric proteins to attenuate mutant SOD1-related toxicity was analyzed by MTS assay using N2a cells. The expression of SOD1^{G85R}, as compared to that of SOD1^{WT}, decreased the viability of cells. Overexpression of Dorfin reversed the toxic effect of SOD1^{G85R}, whereas overexpression of CHIP did not. Dorfin-CHIP^{L} had a significantly greater rescue effect on SOD1^{G85R}-related cell toxicity than did Dorfin (Fig. 7A). We also measured the cell viability of N2a cells overexpressing Mock, Dorfin, and Dorfin-CHIP^{L} with various amounts of constructs, and found no difference in toxicity among them (Supplementary Fig. 2).

A structure that Johnston et al. (1998) called aggresome is formed when the capacity of a cell to degrade misfolded proteins is exceeded. The accumulation of mutant SOD1 induces visible macroaggregation, which is considered to be 'aggresome' in N2a cells. We examined the subcellular localizations of Dorfin, CHIP, and Dorfin-CHIP^{L} by immunostaining N2a cells expressing SOD1^{G85R}-GFP. Dorfin was localized in aggresomes with substrate proteins, as in our previous studies. Dorfin-CHIP^{L} was also seen in aggresomes, whereas the staining of CHIP was diffusely observed in the cytosol (Fig. 7B). We counted these visible aggregations with or without MG132 treatment. Dorfin decreased the number of aggregation-containing cells, as has been reported (Niwa et al., 2002), but Dorfin-CHIP^{L} did so more effectively. These effects were inhibited by the treatment of MG132 (Fig. 7C).

Discussion

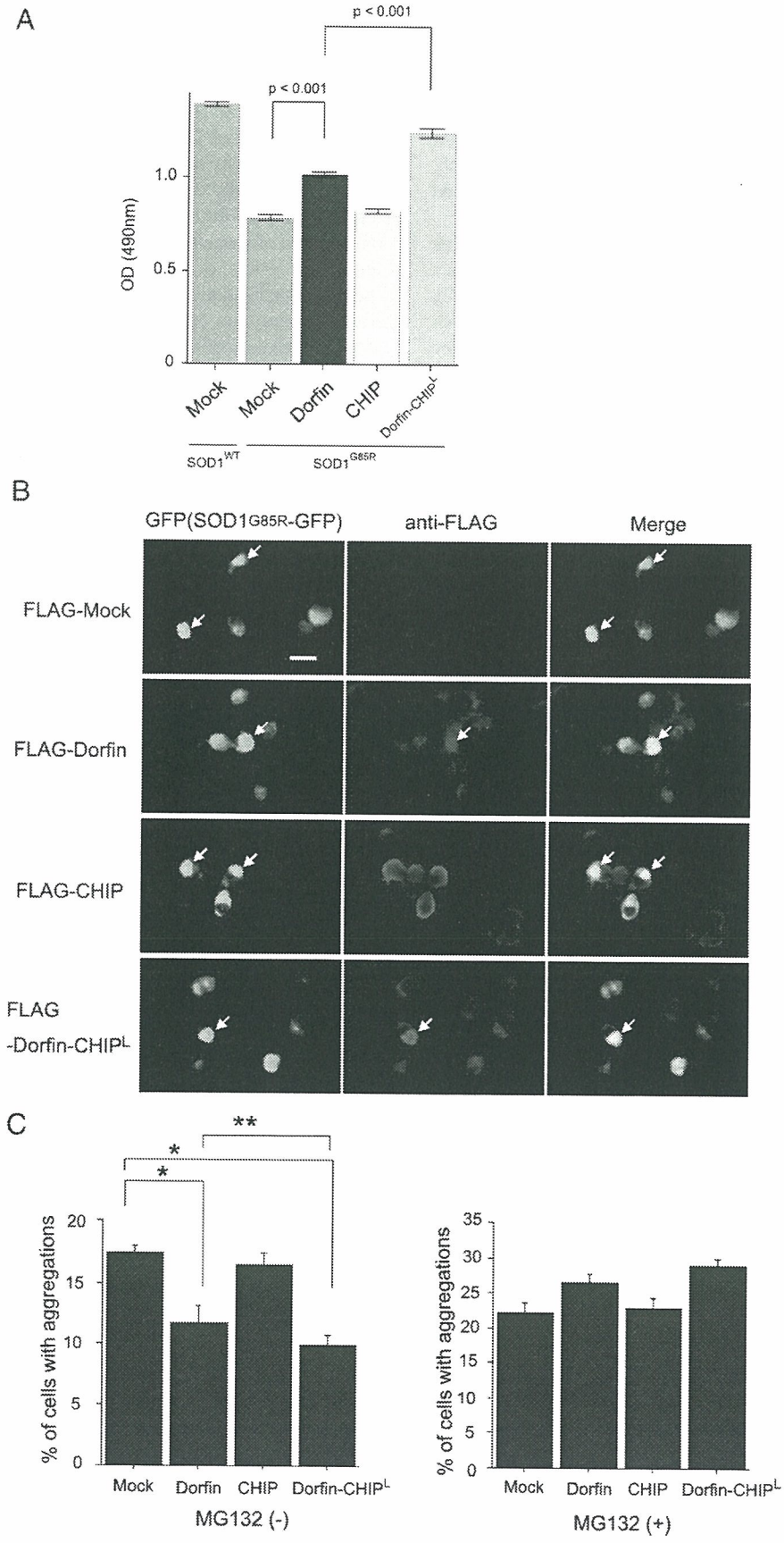
E3 proteins can specifically recognize and degrade accumulating aberrant proteins, which are deeply involved in the pathogenesis of neurodegenerative disorders, including ALS (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). For this reason, E3 proteins are candidate molecules for use in developing therapeutic technology for neurodegenerative diseases. Dorfin is the first E3 molecule that has been found specifically to ubiquitylate mutant SOD1 proteins as well as to attenuate mutant SOD-associated toxicity in cultured neuronal cells (Niwa et al., 2002).

NEDL1, a HECT type E3 ligase, has also been reported to be a mutant SOD1-specific E3 ligase and to interact with TRAPδ and dvl1 (Miyazaki et al., 2004). It has also been reported that ubiquitylation of mutant SOD1-associated complex was enhanced by CHIP and Hsp70 in vivo (Urushitani et al., 2004). CHIP ubiquitylated Hsp70-holding SOD1 complexes and degraded mutant SOD1, but did not directly interact with mutant SOD1 (Urushitani et al., 2004). Among these E3 molecules, Dorfin seems to be the most potentially beneficial E3 protein for use in ALS therapy since it is the only one that has been demonstrated to reverse mutant SOD1-associated toxicity (Niwa et al., 2002). Furthermore, Dorfin has been localized in various ubiquitin-positive inclusions such as Lewy bodies (LB) in PD, as well as LB-like inclusions in sporadic ALS and glial cell bodies in multiple-system atrophy. These findings indicate that Dorfin may be involved in the pathogenesis of a broad spectrum of neurodegenerative disorders other than familial ALS (Hishikawa et al., 2003; Ito et al., 2003; Ishigaki et al., 2004).

The half-life of Dorfin^{WT} is, however, less than 1 h (Fig. 1, Table 1). The amount of Dorfin is increased in the presence of MG132, a proteasome inhibitor, indicating that Dorfin is immediately degraded in the UPS. Since the nonfunctional RING mutant form of Dorfin, Dorfin$^{C132S/C135S}$, degraded more slowly than did Dorfin^{WT}, Dorfin seemed to be degraded by auto-ubiquitylation. The degradation of Dorfin$^{C132S/C135S}$ is also inhibited by MG132, suggesting that it is degraded by endogenous Dorfin or other E3s. This immediate degradation of Dorfin is a serious problem for its therapeutic application against neurodegenerative diseases.

Several reports have shown that engineered chimera E3s are able to degrade certain substrates with high efficiency. Protac, a chimeric protein-targeting molecule, was designed to target methionine aminopeptidase-2 to Skp1-Cullin-F box complex (SCF) ubiquitin ligase complex for ubiquitylation and degradation (Sakamoto et al.,

Fig. 7. Dorfin-CHIP chimeric proteins can attenuate toxicity induced by mutant SOD1 and decrease the formation of visible aggregation of mutant SOD1 in N2a cells. (A) N2a cells were grown in 96 collagen-coated wells (5000 cells per well) and transfected with 0.15 μg of SOD1^{WT} and 0.05 μg of Mock or 0.15 μg of SOD1^{G85R} and 0.05 μg of Mock, Dorfin, CHIP, or Dorfin-CHIP^{L}. After the medium was changed, MTS assays were done at 48 h of incubation. Viability was measured as the level of absorbance (490 nm). Values are the means± SE, n=6. Statistics were carried out by one-way ANOVA. There were significant differences between SOD1^{G85R}-expressing cells coexpressed with Mock and SOD1^{G85R}-expressing cells coexpressed with Dorfin ($p<0.001$), as well as between SOD1^{G85R}-expressing cells coexpressed with Dorfin and SOD1^{G85R}-expressing cells coexpressed with Dorfin-CHIP^{L} ($p<0.001$). (B) N2a cells were transiently expressed with SOD1^{G85R}-GFP and Mock, Dorfin, CHIP, or Dorfin-CHIP^{L}. **Immunostaining with anti-FLAG antibody revealed that Dorfin, CHIP, and Dorfin-CHIP^{L}** were localized with SOD1^{G85R}-GFP in macroaggresomes (arrows). Scale bar=20 μm (C) The visible macroaggregations in N2a cells expressing both SOD1^{G85R}-GFP and Mock, Dorfin, CHIP, or Dorfin-CHIP^{L} with or without MG132 treatment were counted and the ratio of cells with aggregations to those with GFP signals was calculated. Values are the means± SE, n=4. Statistics were done by one-way ANOVA. *$p<0.01$ denotes a significant difference between cells with Mock and Dorfin or Dorfin-CHIP^{L}. **$p<0.05$ denotes a significant difference between cells with Dorfin and Dorfin-CHIP^{L}.


A
p < 0.001
p < 0.001
OD (490nm)
1.0
0.5
0
Mock
Mock
Dorfin
CHIP
Dorfin-CHIP^L
SOD1^WT
SOD1^G85R
B
GFP(SOD1G85R-GFP)
anti-FLAG
Merge
FLAG-Mock
FLAG-Dorfin
FLAG-CHIP
FLAG
-Dorfin-CHIPL
C
**
*
*
% of cells with aggregations
20
15
10
5
0
Mock
Dorfin
CHIP
Dorfin-CHIP^L
MG132 (-)
35
30
25
20
15
10
5
0
MG132 (+)

2001, 2003). Oyake et al. (2002) developed double RING ubiquitin ligases containing the RING finger domains of both BRCA and BARD1 linked to a substrate recognition site PCNA. Recently, Hatakeyama et al. developed a fusion protein composed of Max, which forms a heterodimer with c-Myc, and the U-box of CHIP. This fusion protein physically interacted with c-Myc and promoted the ubiquitylation of c-Myc. It also reduced the stability of c-Myc, resulting in the suppression of transcriptional activity dependent on c-Myc and the inhibition of tumorogenesis (Hatakeyama et al., 2005). This indicated that the U-box portion of CHIP is able to add an effective E3 function to a U-box-containing client protein.

We postulated that engineered forms of Dorfin could be stable and still function as specific E3s for mutant SOD1s. Dorfin has a RING/IBR domain in the N-terminal portion (amino acids 1-332), but has no obvious motif in the rest of the C-terminus (amino acids 333–838). In this study, we have demonstrated that the hydrophobic domain of Dorfin (amino acids 333–454) is both necessary and sufficient for substrate recruiting (Fig. 2B). In our engineered proteins, the RING/IBR motif of N-terminal Dorfin was replaced by the UPR domain of CHIP, which had strong E3 activity (Murata et al., 2001). Some of the engineered Dorfin-chimeric proteins, such as Dorfin-CHIP$^{D, G, J}$, and ^{L}, were degraded in vivo far more slowly than was wild-type Dorfin, indicating that they were capable of being stably presented in vivo (Fig. 3). However, Dorfin-CHIP^{G} failed to show strong ubiquitylation activity against SOD1^{G85R} in HEK293 cells. Since Dorfin-CHIP$^{D, J}$, and ^{L} were able to bind to SOD1^{G85R} more strongly than did Dorfin-CHIP^{G}, the binding activity was more important for the E3 activity than for the protein stability.

We next showed that although all of the Dorfin-CHIP chimeric proteins bound to mutant SOD1 in vivo, some of them, such as Dorfin-CHIP$^{B, C}$, and ^{I}, bound less than others (Fig. 4A). In HEK293 cells, Dorfin-CHIP$^{D, E, F, J, K}$, and ^{L} ubiquitylated SOD1^{G85R} more effectively than did Dorfin or CHIP; however, in N2a cells only Dorfin-CHIP^{L} had more effective E3 activity than did Dorfin or CHIP. This discrepancy may be due to differences between HEK 293 and N2a cells which could provide slight different environment for the E3 machinery. Therefore, Dorfin-CHIP^{L} was the most potent of the candidate chimeric proteins in degrading mutant SOD1 in the UPS in neuronal cells. We also showed that Dorfin-CHIP^{L} could specifically bind to and ubiquitylate mutant SOD1s but not SOD1^{WT} in vivo, as Dorfin had done (Niwa et al., 2002; Ishigaki et al., 2004) (Fig. 5). This observation confirmed that the hydrophobic domain of Dorfin (amino acids 333–454) is responsible for mutant SOD1 recruiting.

Pulse-chase analysis using N2a cells showed that Dorfin-CHIP^{L} degraded SOD1^{G85R} and SOD1^{G93A} more effectively than did Dorfin (Fig. 6). This is compatible with the finding that Dorfin-CHIP^{L} had a greater effect than Dorfin did on the ubiquitylation against mutant SOD1. The cycloheximide assay verified that the degradation ability of Dorfin-CHIP^{L} against SOD1^{G85R} was stronger than that of Dorfin or CHIP in HEK293 cells (data not shown).

Dorfin-CHIP^{L} also reversed SOD1^{G85R}-associated toxicity in N2a cells more effectively than did Dorfin (Fig. 7). This therapeutic effect of Dorfin-CHIP^{L} was expected from its strong E3 activity and degradation ability against SOD1^{G85R}. Visible protein aggregations have been considered to be hallmarks of neurodegeneration. Increased understanding of the pathway involved in protein aggregation may demonstrate that visible macroaggregates represent the end-stage of a molecular cascade of steps rather than a direct toxic insult (Ross and Poirier, 2004). Two facts that Dorfin-CHIP^{L} decreased aggregation formation of SOD1^{G85R} and that this effect was inhibited by a proteasome inhibitor should reflect the ability of Dorfin-CHIP^{L} to degrade mutant SOD1 in the UPS of cells.

Based on our present observations, Dorfin-CHIP^{L}, an engineered chimeric molecule with the hydrophobic substrate-binding domain of Dorfin and the U-box domain of CHIP, had stronger E3 activity against mutant SOD1 than did Dorfin or CHIP. Indeed, it not only degraded mutant SOD1 more effectively than did Dorfin or CHIP but, as compared to Dorfin, produced marked attenuation of mutant SOD1-associated toxicity in N2a cells. This protective effect of Dorfin-CHIP^{L} against mutant SOD1 has potential applications to gene therapy for mutant SOD1 transgenic mice because this protein has a long enough life to allow the constant removal of mutant SOD1 from neurons. Since Dorfin was originally identified as a sporadic ALS-associated molecule (Ishigaki et al., 2002b) and is located in the ubiquitin-positive inclusions of various neurodegenerative diseases (Hishikawa et al., 2003), this molecule is an appropriate candidate for future use in gene therapy not only for familial ALS, but also for sporadic ALS and other neurodegenerative disorders.

So far, most reports on engineered chimera E3s have targeted cancer-promoting proteins. Dorfin-CHIP chimeric proteins are the first chimera E3s to be intended for the treatment of neurodegenerative diseases. Since the accumulation of ubiquitylated proteins in neurons is a pathological hallmark of various neurodegenerative diseases, development of chimera E3s like Dorfin-CHIP^{L}, which can remove unnecessary proteins, is a new therapeutic concept. Further analysis, including transgenic overexpression and vector delivery of Dorfin-CHIP chimeric proteins using ALS animal models will increase our understanding of the potential utility of Dorfin-CHIP chimeric proteins as therapeutic tools.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2006.09.017.

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Alleviating Neurodegeneration by an Anticancer Agent

An Hsp90 Inhibitor (17-AAG)

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ABSTRACT: Heat shock proteins (HSPs) that function mainly as molecular chaperones play an important role in the folding and quality control of proteins. Compared with these chaperones, Hsp90 is unique in that it binds to substrate proteins, called Hsp90 client proteins. Hsp90 is involved in the folding, activation, and assembly of its client proteins in association with its co-chaperones. Because numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded by Hsp90 inhibitors, 17-allylamino-17-demethoxygeldanamycin (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. In spinal and bulbar muscular atrophy (SBMA), the pathogenic gene product is polyglutamine (polyQ)-expanded androgen receptor (AR), which belongs to the Hsp90 client protein family and is known to be degraded by 17-AAG. We have recently demonstrated that administration of an anticancer agent 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. The ability of 17-AAG to 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 using a novel anticancer agent 17-AAG has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases.

KEYWORDS: neurodegeneration; polyglutamine; anticancer agent; Hsp90 inhibitor

INTRODUCTION

In chronic neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and polyglutamine (polyQ) diseases, abnormal

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accumulation of disease-causing protein is a commonly observed characteristic.[1] A selective elimination of disease-causing protein would be a promising remedy for neurodegenerative disorders.

Among these neurodegenerative disorders, it is well confirmed that 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.[2] In polyQ diseases, spinal and bulbar muscular atrophy (SBMA) was first identified among these diseases[3] and is characterized by premature muscular exhaustion, slow progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles.[4] 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 14–32, but ranges from 40 to 62 CAGs in SBMA patients.[5] A correlation exists between the number of CAG repeats and disease severity.[6,7] The pathologic features of SBMA are motor neuron loss in the spinal cord and brain stem,[4] and diffuse nuclear accumulations and nuclear inclusions (NIs) containing the mutant AR in the residual motor neurons and certain visceral organs.[8]

Heat shock protein (Hsp)90 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.[9–11] Hsp90 functions in a multicomponent 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.[12] Numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded in the ubiquitin–proteasome system (UPS) by Hsp90 inhibitors. 17-allylamino-17-demethoxygeldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now in clinical trials as a novel molecular-targeted agent for a wide range of malignancies.[13] AR also belongs to the Hsp90 client protein family and is degraded in the presence of Hsp90 inhibitors.[14–16]

We have recently demonstrated that 17-AAG markedly ameliorated polyQ-mediated motor neuron degeneration through degradation of mutant AR.[17] We consider that the ability to facilitate degradation of disease-causing protein would be of value when applied to SBMA and other related neurodegenerative diseases. This review discusses our research findings and other studies, and the clinical application of Hsp90 inhibitors to neurodegenerative diseases beyond neoplastic ones.

GENERATION OF LESS TOXIC HSP90 INHIBITOR, 17-AAG

The most classical Hsp90 inhibitor is geldanamycin (GA), a natural product that was developed as an antifungal agent.[18] Later, GA was also found to have

a potent and selective antitumor effect against a wide range of malignancies.[19] Although GA showed potential as a novel anticancer agent,[20] it was also found to have intolerable liver toxicity.[21] To overcome this GA-induced liver toxicity, scientists at the U.S. National Cancer Institute (NCI) succeeded in developing a new derivative of GA, 17-AAG, that shares its important biological activities[22] but shows less toxicity.[23] Owing to this promising derivative, Hsp90 inhibitors have taken a major developmental leap in their clinical applications, and 17-AAG is now in Phase II clinical trials with encouraging results as an anticancer agent.[24–28]

The antitumor effect of Hsp90 inhibitors is due to their ability to specifically bind to the Hsp90 adenosine 5'-triphosphate (ATP)-binding site, thereby modulating Hsp90 function and proteasomal degradation of Hsp90 client proteins.[29–31] Because numerous oncoproteins were shown to belong to the family of Hsp90 client proteins,[12] Hsp90 inhibitors are expected to become part of a new strategy in antitumor therapy.[20] Hsp90 inhibitors including GA and 17-AAG have been shown to have a higher selectivity for tumor cells compared with general antitumor agents.[32,33] This selectivity is due to the high affinity of 17-AAG for the Hsp90 client oncoproteins when they are incorporated in the Hsp90-dependent multichaperone complex, thereby increasing their binding affinity to 17-AAG more than 100-fold.[34] This high selectivity of 17-AAG to the incorporated Hsp90 client protein eventually minimizes its toxic side effects.[31] We think that this selectivity of Hsp90 inhibitors would also be advantageous for the treatment of neurodegenerative diseases.

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 (FIG. 1A).[16,35–37] As for AR, Hsp90 is essential to maintain its high ligand-binding affinity and its stabilization.[9,14] In practice, Hsp90 inhibitors reduce androgen ligand-binding affinity and induce the degradation of AR.[14,15] We also confirmed 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.[17]

17-AAG'S DEGRADATION OF MUTANT AR DEPENDS ON PROTEASOME

To determine whether or not 17-AAG promotes the degradation of the 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 dimethylsulfoxide (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 did the wild type, suggesting that the mutant AR is more sensitive to 17-AAG than is the wild type. The degradation of wild-type and mutant AR by 17-AAG was completely

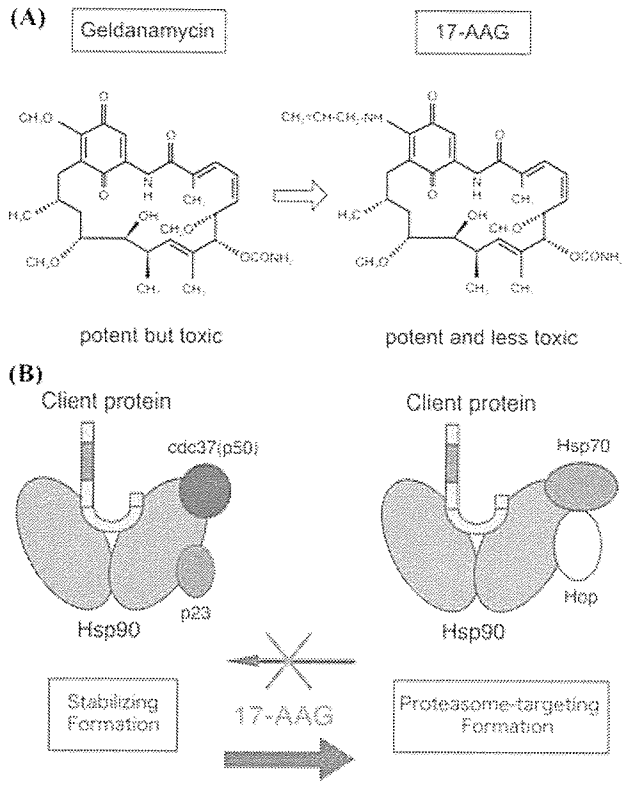

FIGURE 1. Hsp90 inhibitors and the pharmacological effect. (A) Geldanamycin (GA) is the most classical Hsp90 inhibitor. Although GA has a potent and selective antitumor effect against a wide range of malignancies, this agent has intolerable liver toxicity. The 17-AAG, a new derivative of GA, shares its important biological activities but shows less toxicity. (B) The 17-AAG specifically binds ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex. 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 co**mplex toward the stabilizing form and shift it to the proteasomal-targeting form. This figure is modified from a model proposed by Neckers.[16]

blocked by MG132 (FIG. 2A), suggesting that 17-AAG-faciiltated degradation was dependent on the proteasome system as previously reported.[38,39]

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 was 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. 2B), while mRNA levels for both the wild-type and mutant AR were quite similar.[17] These data indicate that 17-AAG preferentially degrades the mutant AR protein without altering mRNA levels.

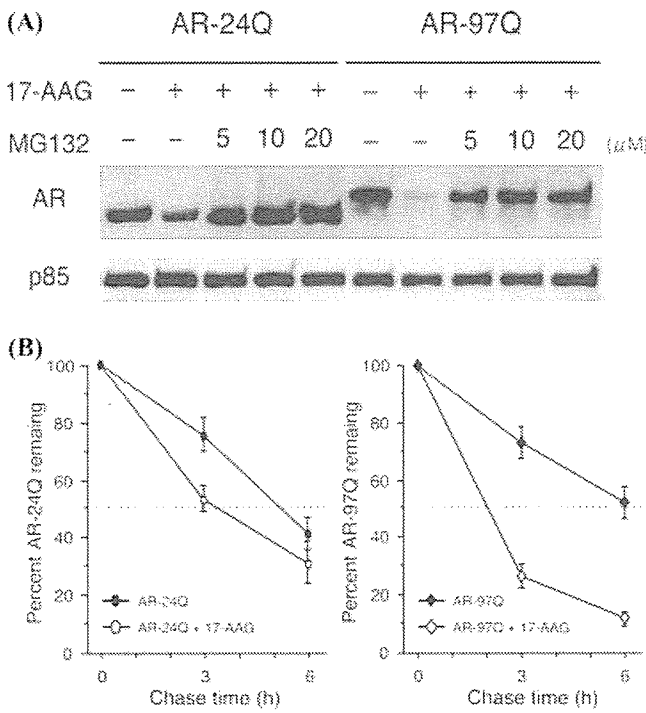

FIGURE 2. The 17-AAG's degradation of mutant AR depends on proteasome function. (A) 17-AAG treatment (36 μM, 6 h) of transfected SH-SY5Y cells reduced the levels of mutant AR (AR-97Q) significantly more than wild-type AR (AR-24Q), however, both decreases were completely blocked by the proteasomal inhibitor, MG132. (B) Effects of 17-AAG on the half-life of wild-type and mutant AR assessed from pulse-chase experiments. Amounts of AR-24Q remaining in the absence and presence of 17-AAG are indicated by closed circles (●) and open circles (○), respectively. 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).

Thus, these in vitro studies indicated that the mutant AR was a good target protein of 17-AAG. To determine whether 17-AAG has the ability to degrade the toxic disease-causing protein 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 WITHOUT DETECTABLE TOXICITY

Referring to previous reports,[15,36,40] we administered 17-AAG thrice a week on alternate days 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). AR-24Q mice treated with 17-AAG displayed no altered phenotypes (data not shown). To evaluate toxic effects of 17-AAG, we examined blood samples from 25-week-old mice treated with 25 mg/kg 17-AAG for 20 weeks. Hematological examination demonstrated that 17-AAG resulted in neither infertility nor liver or renal dysfunction in the AR-97Q male mice at the dose of 25 mg/kg.[17]

When mouse tissues were immunohistochemically stained for mutant AR using the 1C2 antibody, which specifically recognizes expanded polyQ, quantitative analysis revealed a marked reduction in 1C2-positve nuclear accumulation in the spinal motor neurons of the Tg-25 mice compared with those of the Tg-0 mice (FIG. 3B).

Western blot analysis from lysates of the spinal cord 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. 3C). 17-AAG treatments significantly diminished both the high-molecular-weight complex and the monomer of mutant AR in the spinal cord of the AR-97Q mice, whereas they only slightly diminished the wild-type monomeric AR in AR-24Q mice (FIG. 3C). The levels of wild-type and mutant AR mRNA were similar in the respective mice treated with 17-AAG.[17] These observations indicate that 17-AAG markedly reduces not only the high-molecular-weight mutant AR complex but also the monomeric mutant AR protein by preferential degradation of mutant AR.

MOLECULAR-TARGETED THERAPY FOR DISEASE-CAUSING PROTEIN BY AN HSP90 INHIBITOR—BEYOND HSP INDUCER

Hsp90 inhibitors are known to possess the unique pharmacological effect of inducing a stress response, and, in addition to their use as anticancer agents, have also been developed as pharmacological HSP inducers.[41,42] In neurological disorders, many studies have already shown that, taking advantage of **HSP-induction, Hsp90 inhibitors exerted potential neuroprotective effects** in a model of HD,[43–45] tauopathies,[46–48] PD,[49–51] stroke,[52,53] and autoimmune encephalomyelitis.[54] In considering the role for molecular chaperones in neurological disorders, Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases.[55–56] because these chaperones have **the desirable ability to refold abnormal proteins or to carry them to degr**adation as a part of the system of protein quality control.[55–57] However, in our cultured cell models, mutant AR was markedly decreased following 17-AAG treatment even when Hsp70 and Hsp40 induction was completely blocked in the presence of a protein synthesis inhibitor.[17] In SBMA mice, the Western blot analysis

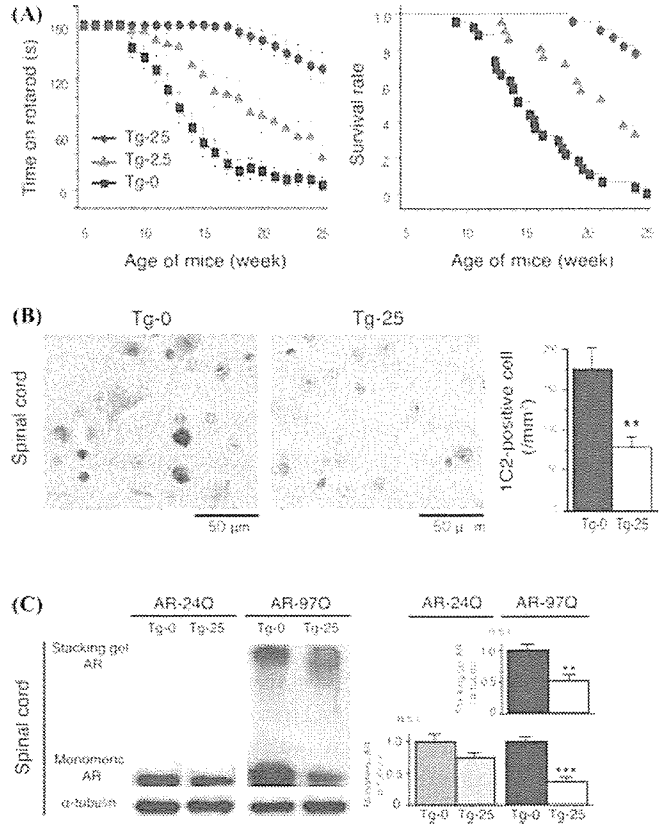

FIGURE 3. Effects of 17-AAG on transgenic SBMA mice. (A) Tg-0, Tg-2.5, and Tg-25 represent AR-97Q mice treated with vehicle alone, 2.5, and 25 mg/kg 17-AAG, respectively (each group: n = 27). The Tg-25 remained longer on the Rotarod than did the Tg-0 mice. A Kaplan–Meier plot shows the prolonged survival 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). **The 17-AAG was less effective at the dose of 2.5 mg/kg than 25 mg/kg in all parameters** tested. (B) Immunohistochemical staining with 1C2 antibody showed marked differences in diffuse nuclear staining and NIs between DMSO-treated mice (Tg-0) and 17-AAG-treated (Tg-25) mice in the spinal anterior horn. There was a significant reduction in 1C2-positive cell staining in the spinal cord (**$P < 0.01$) in the Tg-25 compared with the Tg-0. Values are expressed as means ± SE (n = 6). (C) Western blot analysis of tissue from AR-24Q and **AR-97Q mice probed with an AR-specific antibody. In both spinal cords of mice treated** with 17-AAG, there was a significant decrease in the amount of 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. 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, whereas there was **only a 25.9% decline in the spinal cord of AR-24Q mice. Values are expressed as** means ± SE (n = 5). Statistical differences are indicated by asterisks; *, $P < 0.05$; **, $P < 0.01$.

revealed that the inductions of Hsp70 and Hsp40 were statistically significant, but were also not as pronounced as those arising from genetic manipulation in our previous study.[17,57] 17-AAG thus displayed the limited ability to induce Hsp70 and Hsp40 in mouse tissue, while mutant AR was significantly decreased in Western blot analysis and histopathological study (FIG. 3).

As for SBMA, Thomas et al. have published an interesting report that Hsp90 inhibitor inhibited the aggregation of polyQ-expanded mutant AR in HSF-1 (-/-) mouse embryonic fibroblasts where HSPs were not induced, suggesting that the induction of stress proteins was not necessary for the reduction of mutant AR aggregation by Hsp90 inhibitors.[58] Furthermore, they first demonstrated that Hsp90 inhibitors prevented the aggregation of the mutant AR by the inhibition of Hsp90-dependent trafficking machinery. It seems that Hsp90 would also play a major role in the acceleration of mutant AR aggregation.

Although it would be advantageous for the treatment of neurodegeneration to induce HSPs by Hsp90 inhibitors, considering our research finding in in vivo models, it would be unadvisable to rely only on the induction of nonspecific HSPs for human clinical trials. In SBMA, where it may have its most effective potential, 17-AAG directly accelerates proteasomal degradation of the disease-causing protein, polyQ-expanded AR. 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. We think the induction of HSPs by Hsp90 inhibitors seems to play a supplementary role in neurodegenerative disorders.

CLINICAL APPLICATION OF 17-AAG TO NEUROLOGICAL DISORDERS

In the cultured cells and mouse models of SBMA, we have shown both the efficacy and safety of 17-AAG.[17] Based on our data, 17-AAG would be a candidate for therapeutic agents for SBMA via the ability to facilitate the degradation of mutant protein.

We also confirmed that leuprorelin, a lutenizing hormone-releasing hormone agonist that reduces testosterone release from the testis, significantly rescued motor dysfunction in our SBMA mice.[59] Due to its minimal invasiveness, established in human, and its powerful action, this hormonal therapy has already been in human clinical trials with encouraging results.[60] However, it is an extremely specialized therapy for SBMA and cannot be applied to other polyQ diseases.[59,61,62] In contrast to this hormonal therapy, 17-AAG would be a potential therapeutic agent for SBMA as well as other related diseases.[17] We think that this anticancer agent 17-AAG holds enormous potential for application to a wide range of neurodegenerative diseases in addition to SBMA as previously reported.[41,55,63] For development of Hsp90 inhibitor treatment in neurological disorders, we regard this general versatility as very important. Among neurodegenerative disease-causing proteins, only AR in SBMA

is established as an Hsp90 client protein at this time. However, evidence has accumulated that some Hsp90 client proteins would exert adverse influences on several neurodegenerative disorders.[64–66]

It is well known that many kinases involved in signal transduction belong to the family of Hsp90 client proteins targeted by 17-AAG. Phosphorylated tau is a representative disease-causing protein associated with tauopathies including fronto-temporal dementia, progressive supranuclear palsy, corticobasal degeneration, and multiple system atrophy. Interestingly, phosphorylated tau is a targeted protein of Hsp90 inhibitors.[46] 17-AAG reduces the total amount of phosphorylated tau and its abnormal aggregation. Dou et al. showed that GA and 17-AAG indirectly blocked abnormal tau phosphorylation by inhibition of the Raf-MEK-ERK pathway,[67] of which upstream kinase Raf is also an Hsp90 client protein.[12,68] Extracellular signal-regulated kinase (ERK) is known to mediate the activation and stabilization of phosphorelated tau.[69,70] Along these same lines, LaFevre-Bernt & Ellerby 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.[71] 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 that ERK activation is an important executor of neuronal damage.[72–75] 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. For further development of Hsp90 inhibitors beyond malignancies, it is of considerable importance to assess whether or not other Hsp90 client proteins would exert an adverse effect on neurological disorders.

INTEGRATION OF NEURONAL AND NEOPLASTIC DISORDERS

Most antitumor agents that have been therapeutically applied to neurodegenerative diseases[76,77] have some cytotoxic effects on normal cells, which must be overcome in any clinical application against neurodegeneration. Because neurodegenerative diseases generally follow a chronic progression and the medical treatment is long-standing compared with that for malignancy, the toxic side effects should be extensively suppressed. In contrast to general antitumor agents, the effects of 17-AAG have been known to have a high selectivity to tumor cells that would yield desirable results for neurodegenerative diseases.[32,33]

Considering our research findings and those of the other above-mentioned reports, in addition to its role in malignancies, Hsp90 client protein exerts an adverse influence on the nervous system in some situations. In this case,

it is reasonable to consider modulating Hsp90 function appropriately, where 17-AAG would exert the maximum pharmacological effect.

Until now, when considering the role of molecular chaperones in neurological disorders, Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases.[55,56] However, evidence has accumulated that Hsp90 would be an important therapeutic target in neurodegenerative disorders.[17,58,67] We believe that 17-AAG, an Hsp90 inhibitor, has great potential to become a new molecular-targeted therapy against a wide range of neurodegenerative diseases as well as malignancies. It is not too much to say that neuronal and neoplastic disorders have something in common, so crossover researches between them should be performed for their further development.

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Gene Expression Profiling toward Understanding of ALS Pathogenesis

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ABSTRACT: Although more than 130 years have gone by since the first description in 1869 by Jean-Martin Charcot, the mechanism underlying the characteristic selective motor neuron degeneration in amyotrophic lateral sclerosis (ALS) has remained elusive. Modest advances in this research field have been achieved by the identification of copper/zinc superoxide dismutase 1 (SOD1) as one of the causative genes for rare familial ALS (FALS) and by the development and analysis of mutant SOD1 transgenic mouse models. However, in sporadic ALS (SALS) with many more patients, causative or critical genes situated upstream of the disease pathway have not yet been elucidated and no available disease models have been established. To approach genes causative or critical for ALS, gene expression profiling in tissues primarily affected by the disease has represented an attractive research strategy. We have been working on screening these genes employing and combining several new technologies such as cDNA microarray, molecular indexing, and laser capture microdissection. Many of the resultant genes are of intense interest and may provide a powerful tool for determining the molecular mechanisms of ALS. However, we have barely arrived at the starting point and are confronting an enormous number of genes whose roles remain undetermined. Challenging tasks lie ahead of us such as identifying which genes are really causative for ALS and developing a disease model of SALS with due consideration for the expression changes in those genes.

KEYWORDS: ALS; SOD1; gene expression analysis; cDNA microarray; molecular indexing; laser capture microdissection

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative and fatal human disorder characterized by loss of motor neurons in the spinal cord, brain stem,

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and motor cortex, presenting as weakness of the limbs, speech abnormalities, and difficulties in swallowing.[1] The terminal phases of the disease involve respiratory insufficiency and half of the patients die within 3 years after the onset of symptoms. ALS can be inherited as an autosomal dominant trait in a subset of individuals who make up 5% to 10% of the total population of those affected. In addition, 20% to 30% of familial ALS (FALS) cases are associated with a mutation in the copper/zinc superoxide dismutase 1 gene (SOD1).[2] However, more than 90% of ALS patients are sporadic, not showing any familial trait. Since there have been no available disease models for sporadic ALS (SALS) as of now, transgenic mouse models or cell culture models[3] of ALS associated with SOD1 mutations have proven very useful in studying the initial mechanisms underlying this neurodegenerative disease of unknown etiology. The use of an animal model makes it possible and easy to investigate the different stages of disease progression including the early preclinical phase.

One of the experimental approaches toward a more comprehensive understanding of the molecular changes occurring in ALS is gene expression study[4] employing array-based methods or a differential display and its related techniques. Using transgenic mouse models expressing the SOD1 gene with a G93A mutation, we performed cDNA microarray analysis[5] to reveal the transcriptional profiles of affected tissues, namely, spinal anterior horn tissues. This analysis revealed an upregulation of genes related to an inflammatory process together with a change in apoptosis-related gene expression at the presymptomatic stage prior to motor neuron death.

Next, we extended our gene expression study from mouse to human postmortem spinal anterior horn tissues obtained from SALS patients. In this analysis, we employed a molecular indexing technique, a modified version of the differential display developed by Kato in 1995.[6] These PCR-based screening procedures have the advantage of being able to cover an unrestricted range of expressed genes including even hitherto unknown ones. As a result, we have successfully cloned a novel gene designated dorfin,"[7] the expression of which was upregulated in SALS spinal cords.

Using spinal anterior horn tissues of SOD1 mutant mice or SALS patients as starting materials, these gene expression studies[5,7] have shed considerable light on the pathogenesis of FALS and SALS. However, in the spinal anterior horn tissues of ALS spinal cords, there are reduced numbers of motor neurons with glial cell proliferation. The alteration of the gene expression in the spinal anterior horn tissues could reflect the number of motor neurons and glial cells during disease progression. Such a disadvantage in using anterior horn tissues as starting materials prompted us to try to extract a pure motor neuron-specific gene expression profile. To this end, we employed the technology of laser capture microdissection[8] combined with T7-based RNA amplification and cDNA microarrays, which culminated in the successful detection of a total of 196 genes considered important for the SALS molecular mechanism.[9]