Small Molecule Inducers of Heat-Shock Response Reduce polyQ-Mediated Huntingtin Aggregation

A Possible Therapeutic Strategy

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Abstract

Enhancing cellular defense mechanisms against different kinds of stress may be an attractive therapeutic strategy for neurodegenerative diseases. In particular, inducing the expression of molecular chaperones might reduce the formation of misfolded proteins and toxic aggregates that occur in polyglutamine (polyQ) disorders such as Huntington’s disease. Geldanamycin, a natural substance that modulates Hsp90 function, was previously shown to induce a heat-shock response and to reduce polyQ aggregation in mammalian cells. However, because of toxic and unfavorable pharmacokinetic properties, geldanamycin is not suitable for clinical use. In this study we evaluated the effects of the pharmacologically improved geldanamycin derivatives 17-DMAG and 17-AAG on polyQ aggregation in mammalian cells. Quantitative RT-PCR and SDS-PAGE experiments revealed that 17-DMAG induces expression of the molecular chaperones Hsp40, Hsp70, and Hsp105 in mammalian cells and inhibits the formation of mutant huntingtin aggregates with higher efficiency than 17-AAG or geldanamycin itself. Induction of a heat-shock response and inhibition of polyQ aggregation occurred at nanomolar concentrations. We suggest that geldanamycin derivatives such as 17-DMAG should be considered for the development of a drug treatment for polyQ disorders and other neurodegenerative diseases involving protein aggregation.

Introduction

Polyglutamine (polyQ) diseases are late-onset, progressive neurodegenerative disorders characterized by dysfunction and eventually death of disease-specific neuron populations. Huntington’s disease (HD), the most frequent polyQ disorder, is caused by an unstable CAG trinucleotide repeat expansion located in exon-1 of the IT-15 gene encoding huntingtin (htt), a ubiquitously expressed protein of unknown function [1]. This leads to elongation of the protein’s polyQ domain, which makes htt prone to aggregation in vitro and in vivo [2, 3]. PolyQ disorders are characterized by the formation of intraneuronal inclusion bodies in the brain regions in which neurodegeneration occurs [2]. Whether these microscopically visible inclu-
sions or their soluble precursor aggregates such as oligomers are toxic to cells has been intensely debated [4–7].

Molecular chaperones are part of the cellular defense strategies against different kinds of cell stress. They prevent inappropriate interactions between non-native proteins, enhance the efficiency of de novo protein folding, and promote the refolding of misfolded proteins or target them for proteasomal degradation [8, 9]. They also have been found to localize to polyQ inclusions [8, 10–12]. The chaperones Hsp40 and Hsp70 were shown to suppress protein aggregation in model systems of HD and other glutamine repeat disorders [13, 14]. Several studies have demonstrated that overexpression of Hsp70 and/or Hsp40 can suppress polyQ-mediated neurotoxicity in cultured neurons as well as in Drosophila and mouse models of polyQ diseases [11, 15].

To date, only symptomatic treatments of limited effectiveness are available to patients suffering from these diseases [16, 17]. Enforcing cellular defense mechanisms against misfolded proteins is a promising therapeutic approach. Different strategies have been proposed to assist molecular chaperone-based cellular pathways. One of them is the generation of small molecules that modulate the function of Hsp90. Hsp90 is a highly conserved, constitutively expressed protein. It is part of a multicomponent complex that regulates the activities of so-called Hsp90 client proteins, many of which are involved in signal transduction or regulation of gene transcription [18, 19]. Factors that inhibit Hsp90 function lead to decreased activity of regulatory proteins. One of these factors is geldanamycin (GA), a naturally occurring ansamycin benzoquinone antibiotic that was found to exert potent antitumor activity [20]. Close analysis of this activity revealed that GA inhibits Hsp90 function by association with the protein’s ATP-binding site [21–23]. GA was subsequently shown to disassemble the interaction of Hsp90 with the heat-shock transcription factor HSFI, thereby activating a heat-shock response in mammalian cells [24]. Later, inhibition of intracellular aggregation of mutant htt in mammalian cells was demonstrated [25].

Unfortunately, GA is highly hepatotoxic, poorly water soluble, and displays low stability in biological fluids. For these reasons, less toxic chemical derivatives of GA with more favorable pharmacokinetic properties have been developed (fig. 1). 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is a semisynthetic GA derivative that is currently in phase II clinical trials for various cancer types [26]. Its toxicity is much lower than the one of GA [27]. Similar to GA, 17-AAG has to be injected, with a bioavailability after intraperitoneal injection (i.p.) and oral delivery of 99 and 24%, respectively [28]. Compared to GA and 17-AAG, the more water-soluble derivative 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) has an improved bioavailability of 50 and 100% after oral and i.p. delivery, respectively [29, 30]. In this study, we investigated both GA derivatives for their abilities to induce a heat-shock response and modulate polyQ aggregation in mammalian cells.

**Experimental Procedures**

**Cells and Plasmids**

The cloning of the human cDNA encoding htt exon 1 protein with a polyQ stretch of 72 glutamines into pEGFP-C1 (Clontech), giving rise to pEGFP-HD72Q, was described previously [25]. All plasmids used were sequenced. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 10% fetal calf serum and containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Transfection was performed using Lipofectamin Plus reagent according to the manufacturer’s recommendations (Invitrogen GmbH, Karlsruhe, Germany). 17-AAG and 17-DMAG were kindly provided by Koken Biosciences Inc., Hayward, Calif., USA; GA was purchased from Sigma, Munich, Germany. GA, 17-AAG and 17-DMAG were dissolved as 2 mM stock in DMSO, diluted into fresh medium to give final concentrations of 5–40 mM, and added to cells 3 h post-transfection. Control cells were treated with DMSO. 40 h after transfection, the cells were harvested and lysed in the presence of protease inhibitors. Cell lysis was performed as described [25]. For the analysis of cytotoxicity, COS-1 cells were seeded into 96-well plates (10,000 cells/well) and treated with the compounds for 48 h. An XTT assay was performed following the manufacturer’s recommendations (Promega GmbH, Mannheim, Germany). Absorbance of the formazan product at 490 nm was determined using a Synergy HT microplate reader (Biotek, Germany).

**TagMan RT-PCR**

COS-1 cells were seeded in T75 culture flasks and treated with test compounds for 16 h. Cells were lysed and centrifuged in a Qiashredder homogenizer (11,000 g, 2 min). Whole-cell RNA was purified from lysates using Qiagen RNeasy columns (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions including DNase I treatment (Qiagen). 5 μg of total RNA were reverse transcribed using SuperScript III reverse transcriptase and oligo(dT)$_{24}$ primers (Invitrogen, Germany). Pre-designed TaqMan probes and primer pairs were used for detection of Hsp40 (Hs00428680_m1), Hsp70 (Hs00271244_s1), and Hsp105 (Hs00198379_m1) mRNAs. Real-time PCR was performed on cDNA samples in 96-well optical plates on an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany).

**Western Blot Analysis and Filter Retardation Assay**

Proteins were denatured under reducing conditions and separated by SDS-PAGE (12.5%), then transferred to nitrocellulose membranes according to standard procedures. Membranes were
blocked with 3% nonfat dry milk in TBS and incubated with the following antibodies: anti-htt (HD1, diluted 1:5,000), anti-GFP (Roche, diluted 1:1,000), anti-Hsp40 (StressGen Biotechnologies, Victoria, Canada), anti-Hsp70 antibody (1:200, Santa Cruz Biotechnology sc-1060), anti-Hsp90 (1:1,000, Santa Cruz Biotechnology sc-7947), or anti-Hsp105 (1:1,000, Santa Cruz Biotechnology sc-6241). For the filter retardation assay [3, 31], protein samples (1–20 μg) were heated at 98°C for 5 min in 2% SDS and 50 mM DTT and filtered through a 0.2 μm cellulose acetate membrane (Schleicher & Schuell, Germany) using a BRL dot-blot filtration unit. Captured aggregates were detected by incubation with CAG53b IgG (diluted 1:5,000) or anti-GFP antibody followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG and the fluorescent substrate AttoPhos (Europa Bioproducts Ltd., Cambridge, UK). Quantitation of the captured aggregates was performed using a Fuji LAS 2000 Imager (Tokyo, Japan) and the AIDA 1.0 image analysis software.

Fluorescence Microscopy

COS-1 cells were seeded on glass cover slips, grown overnight, and transfected with pEGFP-HD72Q using Lipofectamine Plus reagent. 3 h post-transfection, test compounds were added and the cells were grown for a further 40–44 h. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and cell nuclei counterstained using Bis-benzimide (Sigma, Germany). The samples were examined by direct observation of the green fluorescent fusion protein using an Axioplan-2 fluorescence microscope (Zeiss, Jena, Germany).

Results

In order to test whether the pharmacologically improved GA derivative 17-DMAG (fig. 1) is able to induce heat-shock protein expression, we treated COS-1 cells with different concentrations of the compound. After 16 h, we determined the relative abundances of mRNA transcripts of the heat-shock proteins Hsp40, Hsp70, and Hsp105 by quantitative RT-PCR. 17-DMAG, at a concentration of 5 nM, did not induce an increase in gene transcription of the heat-shock proteins. At 20 nM tran-
scription levels of the chaperones were significantly increased in the presence of 17-DMAG, with the most prominent effect observed for Hsp70 (fig. 2a–c). 17-AAG similarly increased Hsp70 gene transcription (data not shown).

Next, we tested whether this upregulation of heat-shock protein expression could be confirmed in cells expressing mutant htt. COS-1 cells were transfected with a plasmid encoding the aggregation-prone EGFP-HD72Q protein. This protein contains an N-terminal htt fragment corresponding to exon 1 of the HD gene with an elongated polyQ stretch of 72 glutamine residues [25]. The cells were then treated with different concentrations of 17-DMAG. 40 h post transfection cells were lysed and the protein levels of Hsp40, Hsp70, Hsp105, EGFP-HD72Q, and β-actin were determined by western blotting. As shown in figure 3, the relative amounts of the chaperones Hsp40, Hsp70 and Hsp105 increased significantly with higher concentrations of 17-DMAG. No such effect was observed with the proteins EGFP-HD72Q and β-actin as well as Hsp90 (not shown). This indicates that 17-DMAG elicits a heat-shock response in mammalian cells expressing mutant htt. A similar, although weaker increase in Hsp40, Hsp70, and Hsp105 protein levels was found upon 17-AAG treatment (data not shown).

To examine whether induction of Hsp40, Hsp70, and Hsp105 expression by treatment with GA, 17-DMAG and 17-AAG affects mutant htt aggregation, we utilized COS-1 cells expressing EGFP-HD72Q. In untreated cells (DMSO control) SDS-insoluble htt aggregates were detected in the lysates 40 h post transfection using a filter retardation assay [31]. However, when GA, 17-DMAG or 17-AAG were added 3 h after transfection, the amounts of mutant htt aggregates decreased with increasing concentration of the compounds (fig. 4a, b). The IC$_{50}$ values for the inhibition of polyQ aggregate formation were 14 nM, 8 nM and 20 nM for GA, 17-DMAG and 17-AAG, re-
spectively. This indicates that the compound 17-DMAG is the most potent inhibitor of polyQ aggregation in this cell model. The protein level of the mutant htt fragment expressed in these cells was not altered by the compounds (fig. 3), demonstrating that the decrease in polyQ aggregates was not due to reduced levels of mutant htt. The compounds’ cytotoxicity was determined by an XTT assay. No significant toxic effects were observed up to 40 nM (data not shown). Thus, the reduction of mutant htt aggregates in the presence of the Hsp90 inhibitors was not simply attributable to cytotoxicity of the compounds.

The results obtained by the filter retardation assay were confirmed by fluorescence microscopy. As GFP was a component of the fusion protein used, localization of the expressed htt protein could easily be visualized. Typical cells are depicted in figure 5. Control cells were then compared with treated cells. Nearly half of the control cells contained inclusion bodies with or without additional cytoplasmic fluorescence (fig. 5a, b). When cells were treated with increasing doses of 17-DMAG, the fraction of inclusion-containing cells decreased in a dose-dependent manner (fig. 5c). Instead, more cells exhibited diffuse cytoplasmic fluorescence without any inclusions. The concentrations of 17-DMAG at which this effect was observed were nearly identical to the ones that reduced the amounts of SDS-insoluble htt aggregates detected by the filter retardation assay, thereby confirming these results.

**Fig. 5.** Fluorescence microscopy of COS-1 cells expressing EGFP-HD72Q. Transfected COS-1 cells were grown in the presence of Hsp90 inhibitors or solvent DMSO alone, then fixed and examined for GFP fluorescence. Nuclei were stained using bis-benzimide (Hoechst). 40 h after transfection, transfected control cells contained intracellular inclusion bodies with (a) or without additional cytoplasmic fluorescence (b). c Percentages of cells containing one or more inclusion bodies upon treatment with 17-DMAG or solvent DMSO, respectively. Data are presented as mean values ± SEM. Stars indicate statistical significances of * p < 0.01 or ** p < 0.0001, respectively, using Student’s t test. In total, 2,000 cells were counted.
Discussion

Previously, it was shown that GA efficiently reduces the formation of polyglutamine aggregates in mammalian cells [25, 32]. However, due to certain pharmacokinetic and toxic characteristics, GA is not suitable for clinical use. In this study we showed that the pharmacologically more favourable derivatives 17-AAG and 17-DMAG are able to efficiently induce a cellular heat-shock response and reduce the formation of mutant htt aggregates in mammalian cells. While the efficiency of 17-AAG in the inhibition of polyQ aggregate formation was slightly below that of GA, 17-DMAG was even more active than GA. The concentrations required for heat-shock response induction and inhibition of polyQ aggregation were almost identical, suggesting that it was indeed the upregulation of molecular chaperones such as Hsp40, Hsp70, or Hsp105, that prevented the formation of mutant htt aggregates in the presence of Hsp90 inhibitors.

It might be questioned whether the reduction of polyQ aggregate formation is desirable. Several studies have suggested that soluble oligomeric polyQ intermediates rather than large insoluble inclusion bodies might be the most toxic aggregate species in HD [6]. The molecular chaperones Hsp40 and Hsp70 are known to interfere with very early steps in the process of protein misfolding and oligomerization [9, 33]. Therefore, it is likely that not only large insoluble amyloids or inclusions, but also soluble oligomers and precursor aggregates are reduced upon induction of a heat-shock protein response by GA analogues. In accordance with this, GA was shown to enhance Hsp70 expression and to reduce degeneration of dopaminergic neurons in flies transgenic for α-synuclein [34, 35].

In support of the assumption that Hsp90 inhibition is beneficial in the treatment of neurodegenerative diseases, 17-AAG was demonstrated to ameliorate motor impairment in a transgenic mouse model of spinal and bulbar muscular atrophy (SBMA), which is caused by an elongated polyQ domain in the androgen receptor [36]. In this study, soluble and aggregated androgen receptor, a known Hsp90 client protein, was shown to be reduced by 17-AAG in cultured cells as well as in treated mice, suggesting that degradation of mutant AR was accelerated. In contrast, the reduction of mutant htt aggregates observed by us was not due to accelerated degradation of mutant htt as demonstrated by unaltered levels of the protein in spite of 17-DMAG treatment (fig. 3). This suggests that GA derivatives such as 17-AAG and 17-DMAG might be therapeutic in other polyQ disorders as well.

The concentrations sufficient to inhibit polyQ aggregation were surprisingly low, with IC_{50} values of 20 nM for 17-AAG and 8 nM for 17-DMAG, respectively. However, 17-AAG, like GA, is poorly soluble and displays low oral bioavailability. 17-DMAG has several potential advantages over 17-AAG or other GA analogs studied so far. It is water-soluble and has oral bioavailability values that are significantly better than those of 17-AAG. It does not give rise to potentially toxic metabolites [30], and it was reported to exert anticancer activity in vitro and in animal models [37]. A phase I clinical trial using 17-DMAG for cancer treatment has recently been initiated.

Our study suggests that it might be highly worthwhile to test the therapeutic effects of GA analogues in animal models of HD and, possibly, other polyQ disorders.

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