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Cystamine and Intrabody Co-treatment Confers Additional Benefits in a Fly Model of Huntington's Disease

S. F. Bortvedt^{1,2,3}, J. A. McLearn^{2,4}, A. Messer^{2,5}, A. J. Ahern-Rindell¹, and W. J. Wolfgang^{2,5,§}

¹ University of Portland Biology Department, Portland, Oregon 97203, USA

² Division of Infectious Diseases, Wadsworth Center, New York State Department of Health, Albany, New York 12208, USA

⁵ Department of Biomedical Science, School of Public Health, University of Albany, Albany, New York 12201, USA

Abstract

Huntington's disease (HD) is a lethal, neurodegenerative disorder caused by expansion of the polyglutamine repeat in the Huntingtin gene (*HTT*), leading to mutant protein misfolding, aggregation, and neuronal death. Feeding a *Drosophila* HD-model cystamine, or expressing a transgene encoding the anti-htt intracellular antibody (intrabody) C4-scFv in the nervous system, demonstrated therapeutic potential, but suppression of pathology was incomplete. We hypothesized that a combinatorial approach entailing drug and intrabody administration could enhance rescue of HD pathology in flies and that timing of treatment would affect outcomes. Feeding cystamine to adult HD flies expressing the intrabody resulted in a significant, additional rescue of photoreceptor neurodegeneration, but no additional benefit in longevity. Feeding cystamine during both larval and adult stages produced the converse result: longevity was significantly improved, but increased photoreceptor survival was not. We conclude that cystamine-intrabody combination therapies can be effective, reducing neurodegeneration and prolonging survival, depending on administration protocols.

Keywords

combinatorial therapy; neurodegenerative disease; scFv; polyglutamine

INTRODUCTION

HD is an autosomal, dominant, neurodegenerative disorder caused by mutations in the *HTT* gene that result from an expansion of a CAG repeat coding for a polyglutamine (polyQ) track in the N-terminal region of huntingtin (Htt) (Huntington's Disease Collaborative Research Group, 1993). PolyQ expansions of ≥ 36 residues lead to protein aggregation,

[§]To whom correspondence should be addressed. William J. Wolfgang, Ph.D., Wadsworth Center, NYS Dept. of Health, 120 New Scotland Ave. Albany, NY 12208 USA, Tel.(518) 486-1156, Fax (518) 486-7971, wwolfgan@wadsworth.org.

³Current address: Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA;

⁴Current address: Biology Department, Utica College, Utica, New York 13502, USA;

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progressive age-dependent neuronal degeneration in the basal ganglia, and death (Ross and Poirier, 2004). There is currently no effective treatment for HD.

Intrabodies are a relatively new prospective therapy for neurodegenerative diseases (Messer et al., 2009; Miller and Messer, 2005; Southwell et al., 2009; Stocks, 2006; Wang et al., 2008; Wolfgang et al., 2005). An intrabody is a single, stable, polypeptide containing one or both variable antibody regions that binds with high specificity to a target protein (Miller and Messer, 2005). In cell culture, the C4 anti-htt single-chain Fv intrabody (C4-scFv) can maintain solubility of Htt protein by binding specifically to the protein's amino-terminal region and reducing formation of protein aggregates (Lecerf et al., 2001; Miller et al., 2005). In a *Drosophila* HD model (Steffan et al., 2001), C4-scFv decreased mutant Htt aggregation, decreased neurodegeneration, and increased lifespan (Wolfgang et al., 2005).

In the same *Drosophila* model, cystamine reduced neurodegeneration (Agrawal et al., 2005; Apostol et al., 2003; Marsh and Thompson, 2006). Cystamine is also neuroprotective in mouse models of HD (Bailey and Johnson, 2005; Fox et al., 2004; Karpuj et al., 2002; Van Raamsdonk et al., 2005). Cystamine is a competitive inhibitor of tissue transglutaminase (tTG). Therapeutically, cystamine may interfere with tTG-mediated glutamine crosslinking, reducing Htt aggregate formation (Agrawal et al., 2005; Apostol et al., 2003; Bailey and Johnson, 2005; Dedeoglu et al., 2002; Karpuj et al., 2002; Karpuj et al., 1999; Lorand and Conrad, 1984; Van Raamsdonk et al., 2005).

In the fly neither C4-scFv nor cystamine alone were completely effective at abolishing the HD phenotype (Agrawal et al., 2005; Apostol et al., 2003; Wolfgang et al., 2005). Previous studies suggest that additional therapeutic benefit can be achieved when various drugs are combined, perhaps through correcting multiple cellular pathologies associated with HD (Agrawal et al., 2005; Morton et al., 2005; Ryu et al., 2006; Sarkar et al., 2008; Schilling et al., 2001; Stack et al., 2006; Yang et al., 2009). These studies, however, did not explore the effect of timing of treatment administration. Treatment timing is highly relevant in humans because presymptomatic treatment is an option, due to HD's late onset (in most cases) and availability of accurate, predictive genetic diagnosis. Thus, we hypothesized that a combined treatment would result in additional protection, and that timing of treatment would affect outcomes. We report that a combination of cystamine and intrabody therapies produced an additional therapeutic benefit compared to either treatment alone, and that the timing of cystamine exposure results in differential effects on neurodegeneration and longevity. Finally, the study validates the use of cystamine with intrabody treatment, and is the first to explore the promising option of combining these therapies to treat HD.

MATERIALS & METHODS

Drosophila stocks

Flies were maintained on standard cornmeal media and all experimental crosses were performed at 26°C. The P{w[+mC] = GawB}elav[C155] (*elav*-Gal4) flies were obtained from the Bloomington Stock Center, Indiana University. The UAS-htt exon-1-Q93 flies carry the complete exon 1 from *HTT*, which contains a 93 polyQ repeat expansion (Steffan et al., 2001). The UAS-C4-scFv flies were engineered by standard transgenic methods (Wolfgang et al., 2005).

Drosophila crosses and drug treatment

Male *elav*-Gal4 flies were crossed to virgin females homozygous for the UAS-htt-exon-1-Q93 or for UAS-C4-scFv; UAS-htt-exon-1-Q93. Female progeny expressing Htt or Htt and C4-scFv were used for all experiments. For drug treatment of adult flies, the larvae were raised on standard medium and then transferred to drugged medium within 24 hr after

eclosion. For drug treatment of larvae and adults, eggs were collected on drugged medium for 2–3 days. Larvae were allowed to hatch and maintained on the same medium through metamorphosis. After eclosion adults were transferred to fresh food with the same dose of cystamine.

Survival analysis

Twenty female flies per vial, age 0–18 hr after eclosion, were placed on media containing the predetermined cystamine dose. Flies were transferred onto fresh food every 2–3 days, and vials were maintained at 26°C. Dead flies were recorded daily. Data were analyzed, and survival curves were generated, by the Kaplan-Meier survival analysis method. Statistical significance of differences was determined with log rank statistics (SPSS, Chicago). Significant findings represent a p value ≤ 0.01 .

Pseudopupul assay for neurodegeneration

Fifteen female flies for each experimental condition (age 0–18 hr after eclosion) were placed in vials of standard medium containing the drug at the dosage to be tested and were maintained at 26°C. On day 6, the number of visible rhabdomeres (photoreceptors) in at least 25 ommatidia were counted for five flies of each genotype and drug dose ($n=127-251$). The percent rescue was determined for each condition tested as follows: $100 \times (R_t - R_c)/(7 - R_c)$, where R_t and R_c are the average number of rhabdomeres/ommatidium in the treated and untreated HD flies, respectively (Agrawal et al., 2005). The formula normalizes the percent rescue to number of photoreceptors lost from the untreated flies ($7 - R_c$). Therefore deleterious treatments will produce negative values for the percent rescue. Statistical significance was determined from the raw data using a nonparametric Mann-Whitney (Wolfgang et al., 2005). Significant findings represent a p value ≤ 0.01 .

Cystamine

Standard cornmeal medium was melted, and cystamine (Aldrich C121509) was added to generate 50 μM , 100 μM , and 250 μM doses. A no-drug control (0 μM) of standard medium was used. Drug administration was as described above.

RESULTS

Our studies employ an HD fly model harboring a UAS-htt exon 1 with a 93 polyQ repeat (Steffan et al., 2001) that was expressed throughout the CNS from early embryogenesis onward by means of the *elav*-Gal4 driver. Co-expression of UAS-C4-scFv along with UAS-htt exon 1 reduces HD pathology (Wolfgang et al., 2005). In this scenario, both C4-scFv and the htt transgene are present exclusively in the CNS at all life stages of the fly. By contrast, since cystamine is delivered in the food, we took the opportunity to manipulate timing, as well as dose of administration. Because the disease state in humans can be determined genetically long before the manifestation of symptoms, the opportunity exists to treat individuals presymptomatically. In the fly we can treat with cystamine prior to appearance of symptoms (presymptomatically) by feeding the drug to both larval and adult stages or after the onset of symptoms (postsymptomatically) by adding the drug to the food only during the adult stage. In this way, we have tested the efficacy of cystamine therapy when provided either pre- or postsymptomatically.

Combination therapy confers an additional benefit in slowing photoreceptor neurodegeneration only when cystamine is given postsymptomatically

A number of previous studies in fly models of neurodegenerative disease demonstrate that loss of photoreceptors, an easily quantifiable phenotype, provides an excellent surrogate for

loss of neurons in the CNS (Agrawal et al., 2005; Marsh et al., 2003). Within 18 hr of adult eclosion, HD or HD flies expressing C4-scFv intrabody were transferred to diet containing 0 μ M, 50 μ M, 100 μ M, or 250 μ M cystamine, and the extent of photoreceptor cell degeneration was assayed 6 days later (see Materials and Methods). Earlier studies demonstrated the doses of cystamine employed are not toxic to wild type flies (Apostol et al., 2003). For adult HD flies continuously treated with cystamine alone over the 6 day period (postsymptomatic treatment), only the 100 μ M dose produced a significant 17% rescue of photoreceptors compared to no-drug HD controls, while the 50 and 250 μ M doses had no significant effect (throughout this report significant findings represent a p value ≤ 0.01 ; Fig 1, black bars). In HD flies expressing the C4-scFv intrabody transgene alone, photoreceptors were significantly rescued by 48% compared to no intrabody controls, similar to results of earlier studies (Wolfgang et al., 2005). The combination of the C4-scFv and postsymptomatic 100 μ M cystamine treatments produced an additional benefit, rescuing 66% of photoreceptors compared to HD-controls undergoing neither treatment. Importantly, this represents a significant improvement over either treatment alone (Fig 1). Neither the 50 μ M nor the 250 μ M cystamine dose combined with the C4-scFv intrabody treatment showed any significant difference from intrabody treatment alone.

Next, we wanted to determine if presymptomatic administration of cystamine, by treating throughout larval and adult stages, could further improve photoreceptor rescue either in the presence or absence of intrabody. We found that feeding cystamine during all larval and adult stages was not effective against neurodegeneration of photoreceptors; instead, a pronounced neurotoxicity was observed in these drug-treated HD flies in the absence of intrabody (Fig. 1, white bars). In the presence of the C4-scFv intrabody, cystamine did not impair C4-scFv rescue of photoreceptors, but the combination conferred no additional benefit.

Combination therapy confers a survival benefit only when given presymptomatically

Using Kaplan-Meier survival analysis (see Materials and Methods), we measured mean and median survival time of adults (in days) in cohorts of HD flies treated with cystamine, intrabody, or a combination of the two. Feeding of cystamine to adult HD flies (postsymptomatic treatment) did not significantly improve survival; the highest dose (250 μ M) in fact produced a small yet significant decrease in survival (Table 1). C4-scFv treatment alone produced a significant increase in mean survival, by almost three days, as previously reported (Wolfgang et al., 2005). Combination of either 50 μ M or 250 μ M dose with intrabody treatment led to significantly decreased survival compared to HD-flies treated with C4-scFv alone. When C4-scFv treatment was combined with the 100 μ M (adult) dose of cystamine, survival times were unchanged compared to intrabody treatment alone. Thus, 100 μ M cystamine added to the C4-scFv treatment did not significantly impair the ability of C4-scFv to rescue fly survival, but the combination produced no additional benefit. We then explored the effect on survival of treating with cystamine presymptomatically by extending cystamine treatment to the larval as well as adult stages. The 100 μ M dose of cystamine significantly increased survival of HD flies, relative to survival in no-drug HD controls (Table 2). As before, C4-scFv treatment alone conferred significantly improved survival.

When cystamine was administered at larval and adult stages in conjunction with the intrabody, an additional improvement in survival was observed at the 50 μ M and 100 μ M doses, but not at the 250 μ M dose. In summary, treatment with cystamine during larval and adult stages in combination with intrabody treatment provided an additional improvement in survival but (as shown in the preceding section) no improvement for neuronal survival.

DISCUSSION

When 100 μ M cystamine is fed postsymptomatically to adult HD flies, photoreceptor neurodegeneration is reduced, as previously reported (Agrawal et al., 2005; Apostol et al., 2003). When this dosage of cystamine was combined with C4-scFv treatment, neuronal survival was improved over either treatment alone; however, no significant increase in adult lifespan was associated with postsymptomatic treatment, and in some cases doses other than 100 μ M, actually resulted in reduced survival. Interestingly, presymptomatic treatment with 100 μ M cystamine increased the lifespan of the HD fly and, when combined with C4-scFv, further improved survival. Combining presymptomatic cystamine treatment with C4-scFv allowed the effective dose of the drug to be reduced to 50 μ M, a dose that was ineffective when cystamine was used alone. Combination therapies that allow the drug dose to be reduced are of particular interest since, in our model, the narrow dose response range observed reflects the tendency of higher cystamine doses to be toxic in the HD fly.

None of the combined treatment regimens improved both lifespan and neuronal survival. The combined postsymptomatic therapy showed a significant benefit for photoreceptor cell survival, but had no impact on survival of the fly. By contrast, a presymptomatic combination therapy produced a significant increase in the HD fly lifespan, but did not improve photoreceptor rescue. In all our experiments, expression of Htt and C4-scFv is restricted to the CNS from the early stages of neural development onward. Thus, the disease stage-dependent response must result from the time cystamine treatment is initiated. The different outcomes of these two treatment paradigms may reflect variations in the sensitivity of subsets of neurons to cystamine treatment during disease progression. In addition, because cystamine is delivered systemically, the benefits observed may not be the consequence of a direct action on the CNS.

Cystamine treatment has been previously found to increase lifespan and to improve motor activity in mice (Bailey and Johnson, 2005; Dedeoglu et al., 2002; Fox et al., 2004; Van Raamsdonk et al., 2005), but our findings are the first to demonstrate increased longevity in flies. Additionally, other combined drug therapies have been found to confer additive benefit in flies and mice (Agrawal et al., 2005; Morton et al., 2005; Ryu et al., 2006; Sarkar et al., 2008; Schilling et al., 2001; Stack et al., 2006), just as we now show for the first time with the combination of C4-scFv with cystamine. In these cases, the additional therapeutic benefits likely result from the combined treatments targeting distinct pathways.

Underlying mechanisms of HD pathology remain unclear and the role of the protein aggregates is still being explored. Evidence exists for protective roles for visible aggregates, with the toxic moiety being some, as yet unidentified, soluble intermediate conformer (Arrasate et al., 2004; Bodner et al., 2006; Truant et al., 2008). A number of recent studies have implicated the N-terminal 17 amino acids of Htt as a critical element in controlling pathogenicity and aggregation of the expanded Htt protein (Gu et al., 2009; Tam et al., 2009; Thompson et al., 2009; Williamson et al.) validating the targeting of C4-scFv to this region. Thus, the best current evidence suggests that C4-scFv produces its beneficial effect by altering the aggregation process of mutant Htt and reducing levels of a toxic form, which may either be the visible aggregate itself or an oligomeric precursor form.

As a competitive inhibitor of tTG, cystamine may act by interfering with tTG-mediated glutamine crosslinking, reducing Htt aggregate formation. In addition, evidence exists for a number of other therapeutic mechanisms including, increasing neurotrophic factors (BDNF), antioxidant activity, levels of heat shock chaperone proteins, and/or decreasing apoptotic activity (Bailey and Johnson, 2005; Borrell-Pages et al., 2006; Fox et al., 2004; Karpuij et al., 2002; Lesort et al., 2003). Thus, a plausible hypothesis for the combined benefit of the dual

treatment is that C4-scFv alters the process of aggregation such that mutant Htt is more susceptible to one or more of the cystamine-induced activities leading to further loss or inactivation of toxic forms of Htt.

A number of studies point to a model in which chaperones may mediate cystamine's therapeutic assistance of C4-scFv activity. 1) C4-scFv therapeutic activity was improved when the chaperone Heat-shock protein (Hsp) 70 was elevated genetically in the same *Drosophila* model (McLear et al., 2008). 2) Cystamine benefit in HD mice was associated with increased transcription of another chaperone, Hsp40 (Karpuj et al., 2002). 3) The *Drosophila* homolog of Hsp40, dHdj1, when elevated genetically, suppressed HD pathology in the fly (Kazemi-Esfarjani and Benzer, 2000). Thus, a specific hypothesis would posit that cystamine acts through dHdj1 or other chaperones in *Drosophila* to produce the added benefit observed in combination with C4-scFv. Future investigations using the powerful genetic tools available in *Drosophila* and novel small molecules that enhance Hsps should shed light on whether this is correct or if other mechanisms may be at work.

Preclinical studies in mouse models of HD using gene therapy approaches with intrabodies (C4-scFv, EM48-scFv, Hsp1) (Southwell et al., 2009; Wang et al., 2008) (data not shown) and RNAi (Harper et al., 2005) produced promising results in reducing aggregate burden, and/or motor and cognitive defects, sometimes increasing lifespan. A phase 1 trial of cysteamine (the dimer of cystamine) determined tolerable doses in HD patients (Dubinsky and Gray, 2006). Therefore, these reagents warrant serious consideration for future clinical studies. Furthermore, our data support the pursuit of preclinical studies in mice using combinations of these treatments.

Combinatorial therapeutic treatments, entailing neuronal cell-specific gene delivery with a systemically administered small molecule offer the potential for reducing toxic side-effects because drug efficacy is maintained at a lower dose than would otherwise be possible. Although mechanisms of improved efficacy remain unknown, it is likely that they result, at least in part, from combined treatments acting on multiple cell systems, such as aggregation, and protein quality control pathways. Our results also demonstrate the need to consider the time of treatment, as well as dose. Such considerations are relevant to future human treatment strategies, because reliable diagnosis is possible prior to the onset of disease symptoms. Thus, the present study informs and validates for the first time the now promising approach of combining a small molecule therapeutic with a neuronally-targeted anti-huntingtin intrabody to combat HD pathology.

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Figure 1. Percent photoreceptor rescue in six-day old adult HD flies treated with cystamine, intrabody or a combination of both

Postsymptomatic treatment (black columns) with 100 μ M cystamine resulted in a significant (#) rescue of photoreceptors. A further improvement was observed when C4-scFv was combined with this dose of cystamine (*). The combined treatment was significantly better than either treatment alone. Presymptomatic treatment (white columns) was of no additional benefit beyond intrabody alone for the combined therapy. Presymptomatic treatment with cystamine alone appeared to be toxic to photoreceptors resulting in significantly reduced photoreceptor survival at the 50 and 100 μ M doses (+). Dose of cystamine and presence of C4 scFv are indicated at the top of each pair of columns. * = significantly improved photoreceptor survival compared to HD fly treated with C4-scFv alone; # = significantly improved photoreceptor survival compared HD fly without treatment; + significantly reduced photoreceptor survival compared to HD fly without treatment.

Table 1

Postsymptomatic feeding of cystamine with C4-scFv does not improve survival compared to intrabody rescue alone.

Cystamine conc. & presence of C4-scFv	n	Mean	Median	Effect on survival
0 μ M	117	8.9	9	NA
50 μ M	123	8.8	9	= HD
100 μ M	120	8.8	9	= HD
250 μ M	117	8.2	9	↓ HD
C4 alone	122	11.4	12	↑ HD
C4 & 50 μ M	102	10.5	11	↓ C4/HD
C4 & 100 μ M	113	11.3	12	= C4/HD
C4 & 250 μ M	119	10.4	11	↓ C4/HD

NA; not applicable

= HD; no significant difference compared to untreated HD flies.

↓ HD; significantly reduced survival compared to HD flies.

↑ HD; significantly increased survival compared to HD flies. = C4/HD; no significant difference compared to C4 treated HD flies.

↓ C4/HD; significantly reduced survival compared to C4 treated HD flies.

Table 2

Presymptomatic feeding of both 50 μ M and 100 μ M cystamine with C4-scFv increases survival compared to intrabody alone.

Cystamine conc. & presence of C4-scFv	n	Mean	Median	Effect on survival
0 μ M	63	8.1	8	NA
50 μ M	61	8.5	9	= HD
100 μ M	54	9.6	10	↑ HD
250 μ M	54	8.9	9	= HD
C4 alone	58	10.2	11	↑ HD
C4 & 50 μ M	59	12.2	13	↑ C4/HD
C4 & 100 μ M	61	11.7	12	↑ C4/HD
C4 & 250 μ M	64	10.6	11	= C4/HD

NA; not applicable

= HD; no significant difference compared to untreated HD flies.

↑ HD; significantly increased survival compared to HD flies.

= C4/HD; no significant difference compared to C4 treated HD flies.

↑ C4/HD; significantly increased survival compared to C4 treated HD flies.