Identification of combinatorial drug regimens for treatment of Huntington's disease using *Drosophila*

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We explore the hypothesis that pathology of Huntington's disease involves multiple cellular mechanisms whose contributions to disease are incrementally additive or synergistic. We provide evidence that the photoreceptor neuron degeneration seen in flies expressing mutant human huntingtin correlates with widespread degenerative events in the *Drosophila* CNS. We use a *Drosophila* Huntington's disease model to establish dose regimens and protocols to assess the effectiveness of drug combinations used at low threshold concentrations. These proof of principle studies identify at least two potential combinatorial treatment options and illustrate a rapid and cost-effective paradigm for testing and optimizing combinatorial drug therapies while reducing side effects for patients with neurodegenerative disease. The potential for using prescreening in *Drosophila* to inform combinatorial therapies that are most likely to be effective for testing in mammals is discussed.

combinatorial treatments | neurodegeneration

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an expansion of a homopolymeric polyglutamine (polyQ) stretch within the huntingtin protein (Htt) (1). At least eight other neurodegenerative disorders are also caused by polyglutamine repeat expansions (2, 3). The pathology of these diseases seems to be complex and may affect multiple cellular functions and processes including transcription, protein modification and processing, oxidative stress, and mitochondrial function (4, 5). If multiple cellular events are indeed contributing to pathology, administration of several drugs that target different mechanisms might be expected to achieve greater relief from symptoms. Such a regimen might also allow more modest doses of drugs to be used so as to avoid undesirable side effects caused by high drug concentrations.

Combinatorial drug therapy has proven very effective in the treatment of cancers, AIDS, and many other complex human diseases (6–9). For example, combinatorial treatment of AIDS with at least three different drugs has proven to fully suppress HIV replication, allowing immune reconstitution to occur (8). Combinations of compounds can also enhance antitumor activities, leading to synergistic outcomes (10). Additive neuroprotective effects have also been reported in a mouse model of amyotrophic lateral sclerosis (9).

Drosophila transgenic models of neurodegenerative diseases such as HD have proven to be excellent models of these largely dominant human diseases by replicating most of the features of the disease, such as late onset, reduced longevity, neurodegeneration, and impaired motor function (5, 11, 12). Here, we show that expression of mutant human Htt causes widespread degeneration in the *Drosophila* CNS that correlates with degeneration of photoreceptor neurons. We then use photoreceptor neuron degeneration as a sensitive measure of the effects that drugs or genetic manipulations have on pathology, and we use this to explore combinatorial strategies of drug administration to determine whether targeting distinct cellular mechanisms can produce additive or synergistic suppression of pathology. As studies in flies have translated well to mammalian systems (5), these observations identify at least two pharmacologic combinations that are excellent candidates for testing in mammalian systems.

Materials and Methods

***Drosophila* Stocks, Crosses, and Confocal Analysis.** The polyglutamine expressing transgenic stock used in this study is *w*; P{UAS-Httex1p Q93}4F1. These flies were mated with the pan-neuronal elav driver *w*; P{$w^{+mW.hs}$ = GawB}elavC155 or the mushroom body-specific driver *w*; P{$w^{+mW.hs}$ = GawB}OK107. Cultures were raised at 25°C. The coexpression of the GFP and polyQ transgenes facilitates visualization of the effects of transgene expression.

Immunochemistry. Heads and thoraxes of adult flies were prefixed in 4% formaldehyde at room temperature for 1 h, and the brain and ventral cord were dissected in PBS. The tissue was fixed for an additional 10 min in formaldehyde at room temperature. After permeabilization and blocking (Triton X-100 in PBS, 2 h at room temperature, 5% normal goat serum/0.2% Triton X-100 in PBS, 2 h at room temperature), tissues were incubated with primary antibody in blocking solution overnight at 4°C. After washing in PBS, secondary antibody was applied for 2 h at room temperature. The primary antibodies were anti-GFP (1:800 dilution, Chemicon) and anti-elav (1:200 dilution, Iowa Hybridoma Bank). Secondary antibodies were from Jackson ImmunoResearch (1:200 dilution). Every picture is a merged image of a series of confocal Z-slices (Zeiss LSM510). Three-dimensional images of mushroom bodies (see Fig. 1*A*) were generated by using AMIRA software.

Survival Assay. Eggs collected from *elav*-Gal4 males x Httex1p Q93 females were transferred to vials containing standard *Drosophila* food supplemented with 0.5 μM suberoylanilide hydroxamic acid (SAHA), 50 μM cystamine, 10 μM Congo red, 2 μg/ml geldanamycin and 5 μM Y-27632 for threshold concentrations, and 2 μM SAHA, 100 μM cystamine, and 250 μM Congo red for effective concentrations. Survival was calculated as ratio of eclosed adults to the eggs of the same genotype (males or females). For every combination of compounds, at least four vials were scored with 100 eggs in each.

Pseudopupil Analysis. Seven-day-old flies were decapitated and mounted in a drop of nail polish on a microscopic slide. The head was then covered with immersion oil and examined under Nikon

Abbreviations: HD, Huntington's disease; Htt, huntingtin protein; polyQ, homopolymeric polyglutamine; SAHA, suberoylanilide hydroxamic acid.

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EFD-3/Optiphot-2 scope with ×50 oil objective. At least 200 ommatidia in five to eight flies were examined, and the number of visible rhabdomeres was counted for each.

Climbing Assay. Motor function of 7-day-old flies was monitored by their ability to climb in a vertical tube, as adopted from ref. 13. For each condition, 20 individual flies were tested in a marked tube, and the maximum height reached in 10 sec was recorded.

Results

Htt-Induced Neurodegeneration in *Drosophila* Is Widespread. To test and compare the effectiveness of therapeutic strategies, it is necessary to have a quantitative and convenient assay of HD pathology; however, it is also essential that the biomarker used for such assays be reflective of the pathogenic processes in general. A popular assay of degeneration involves counting the photoreceptor neurons of the *Drosophila* compound eye that produce a repeating trapezoidal arrangement of seven visible rhabdomeres (subcellular light-gathering structures) in each ommatidium or facet of the eye (14, 15). Expression of Httex1p Q93 in *Drosophila* neurons leads to the progressive loss of these photoreceptor cells (16), which can be readily observed under a light microscope, and therefore provides a quantitative measure of neuronal degeneration (11, 12). To determine whether loss of photoreceptor neurons is indicative of more widespread degeneration in the CNS, we have examined the effect of Htt expression on the adult brain using confocal imaging.

The fly mushroom body is essential for complex behaviors such as behavioral plasticity, locomotion (17, 18), and learning and memory (19–21). It comprises ≈3,000 Kenyon cells bundled in five pairs of lobes named α, α', β, β', and γ. To determine the effect of Httex1p Q93 on CNS structures, flies coexpressing Httex1p Q93 and GFP using the mushroom body-specific OK107 driver were compared to control flies expressing GFP alone. Changes in the structure of the mushroom body were apparent, and clear evidence of neuronal loss and degeneration was observed (Fig. 1*A*). The α' and β' lobes are mostly missing, the number of fibers in the γ lobe is reduced, and Kenyon cell bodies responsible for these fibers are also reduced. Quantification of changes in the mushroom body volume was assessed in a double transgenic line of Httex1p Q93 and *dnc*-GFP (a *dunce* GFP fusion protein expressed in all Kenyon cells and the mushroom body). Pan-neuronal expression of Httex1p Q93 results in ≈24% volume reduction in mushroom bodies 10 days after eclosion (Fig. 1*B*). We also examined degeneration in other structures identified by OK107. The number of cells in the median bundle cluster is reduced from ≈20 to ≈8 when Httex1p Q93 is expressed (Fig. 1*C*). In addition, the cells of the ventral ganglia that normally display regular repeating patterns of fibers and cell body locations show a reduced number of cell bodies, and the projections reveal clear dysmorphology (Fig. 1*D*). Cells in the abdominal region of the ventral ganglia also show changes in neuronal morphology with loss of projections and rounded shape, rather than stellate, as observed at high magnification (Fig. 1*E*). These observations demonstrate that pathology occurs throughout the nervous system including the eye which can be easily quantitated, thus validating photoreceptor loss as a marker for general neuronal loss.

Dosage and Toxicity Studies. Because identification of potentially therapeutic compounds usually relies on various surrogate biomarkers that are hypothesized to be relevant to pathology (e.g., reduction of protein aggregates), *in vivo* demonstration of pathogenic relief must be obtained. To establish a foundation for interpreting combinatorial drug-feeding experiments, we determined dosage and toxicity profiles for selected compounds.

We initially focused on Congo red, cystamine, and SAHA for

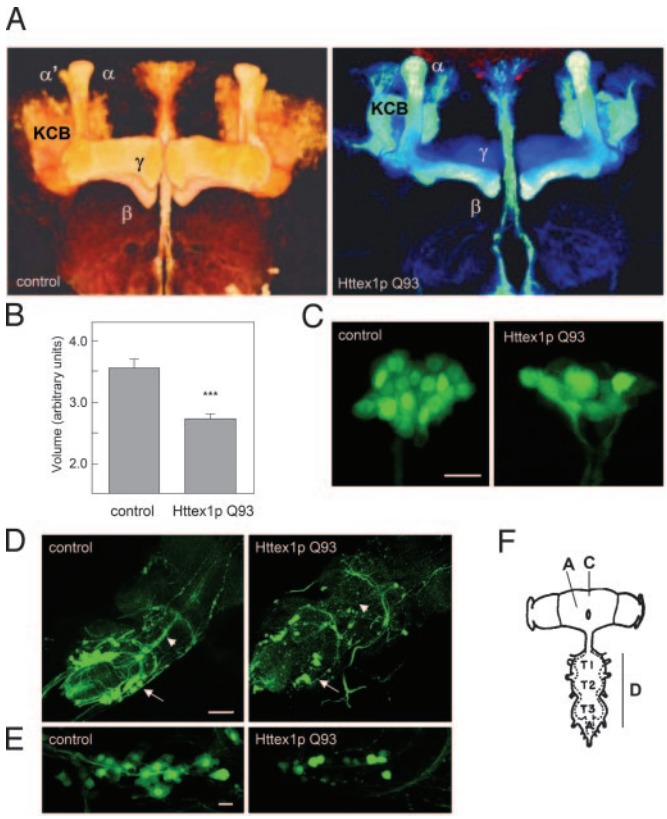

Fig. 1. Htt-induced neuronal degeneration is widespread. (*A*) Mushroom bodies of control (OK107>GFP) and polyQ flies (OK107>GFP; Httex1p Q93). The α' and β' (not visible) lobes are completely missing, and the γ lobe neuropil density is dramatically reduced, as are the number of Kenyon cell bodies (KCB). (*B*) Volumetric analysis of mushroom bodies in flies bearing the UAS>Httex1p Q93 transgene and a *dunce* GFP fusion protein expressed in all Kenyon cells and the mushroom body crossed to *elav*>Gal4. Quantification of the volume of the mushroom bodies in 10- to 14-day-old females indicates that Httex1p Q93 flies show ≈24% volume reduction in mushroom bodies compared with control (***, $P < 0.001$). (*C*) The median bundle group of neurons in central brain of OK107>GFP flies exhibits ≈50% cellular loss when Httex1p Q93 is expressed. At 13 days, controls show 20.5 ± 2.0 SE, whereas experimentals show 8.5 ± 0.4 SE. (Scale bar, 10 μm.) (*D*) Populations of neurons and neurites in the thoracic and abdominal regions of ventral nerve cord exhibit extensive loss of cell bodies (arrows) and loss and disorganization of neuronal projections (arrowheads). (Scale bar, 50 μm.) (*E*) Higher magnification of cell bodies of ventral nerve cord marked with arrows on *D*. (Scale bar, 10 μm.) (*F*) Schematic of the adult brain showing approximate location of structures discussed in *A*–*D*.

proof of principle testing using dosages that we had previously shown to effectively suppress pathogenesis in flies expressing human Httex1p Q93 (16, 22). Importantly, these effective concentrations do not suppress pathology simply through down-regulation of transgene expression as revealed by Northern blot analysis (data not shown). We tested for the maximum tolerable dose of individual drugs, as well as the maximal tolerated concentration of the DMSO carrier. Survival/viability was monitored at various concentrations and compared to flies raised on normal food without drugs. Food containing increasing concentrations of DMSO reveals a toxicity threshold above which survival is reduced (Fig. 2*A*). This barrier limits the highest doses of drugs that can be delivered to flies without toxic effects due solely to solvent concentrations. We also found that cystamine exhibits increasing toxicity over the range tested (up to 500 μM), whereas little toxicity was observed for Congo red and SAHA until doses significantly higher than the effective concentrations

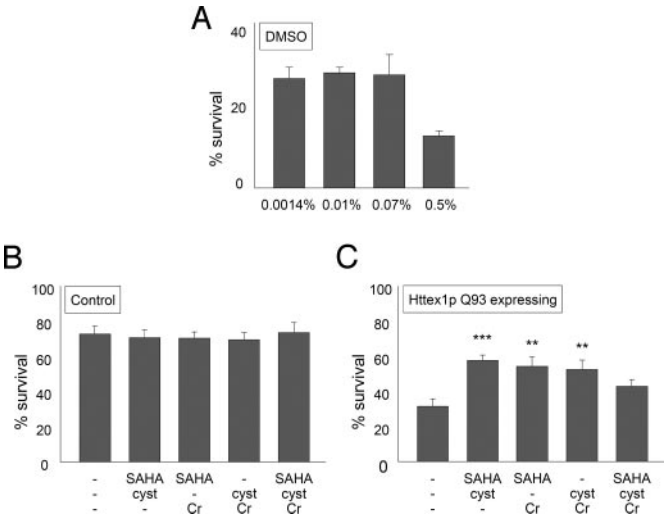

Fig. 2. Toxicity and dosage studies. (*A*) Toxicity of the DMSO carrier. Httex1p Q93-expressing embryos were transferred into vials containing standard fly food supplemented with increasing concentrations of DMSO. A significant reduction in viability was observed with the highest concentration of DMSO tested. (*B*) Compound combinations at low concentration on adults not expressing Httex1p Q93 showed no change in survival. (*C*) Httex1p Q93-expressing siblings, however, showed significant rescue of lethality as compared to control DMSO food. ***, $P \ll 0.001$; **, $P < 0.01$.

are administered (e.g., 100 μM for SAHA and 2.5 mM for Congo red; data not shown).

The goal of this study is to identify the lowest concentrations of drugs that, in combination, can suppress polyglutamine-induced pathology at a level comparable to or better than the single compound feeding at effective concentrations. After feeding flies a range of concentrations of the individual compounds, we identified the "threshold" concentrations as 0.5, 50, and 10 μM for SAHA, cystamine, and Congo red, respectively. These concentrations produced no detectable improvement in survival, photoreceptor neuron degeneration, or motor function induced by expression of Httex1p Q93, if administered alone. Survival of control flies not expressing Htt fed either single or combinations of drugs at threshold concentrations was similar to DMSO-fed flies (Fig. 2*B*), indicating that combining the compounds at these doses does not result in detectable toxicity.

Combination Therapy at Threshold Concentrations Improves Survival of HD Flies. To determine whether combinations of the threshold concentrations can improve survival, double and triple combinations were tested in flies expressing Httex1p Q93. Combinations of compounds at threshold concentrations significantly rescue the polyQ-induced lethality compared to control flies raised in food with DMSO only or fed single compounds (Fig. 2*C*). Notably, pairs of compounds resulted in better survival than that seen in animals fed a mixture of three compounds, possibly indicating additive deleterious effects. Combinations of the higher effective doses of these compounds show less improvement than single effective doses or combinations at threshold concentrations (data not shown). We conclude that pretesting drug combinations in *Drosophila* can reveal potentially effective drug combinations as well as possible deleterious interactions among pharmacologic agents.

Combination Therapy at Threshold Concentrations Effectively Suppresses Neuron Degeneration in a *Drosophila* HD Model. The effect of threshold combinatorial drug feeding on neurodegeneration was evaluated. At threshold concentrations of single compounds, no rescue of neurodegeneration is observed (Fig. 3*A*). At single

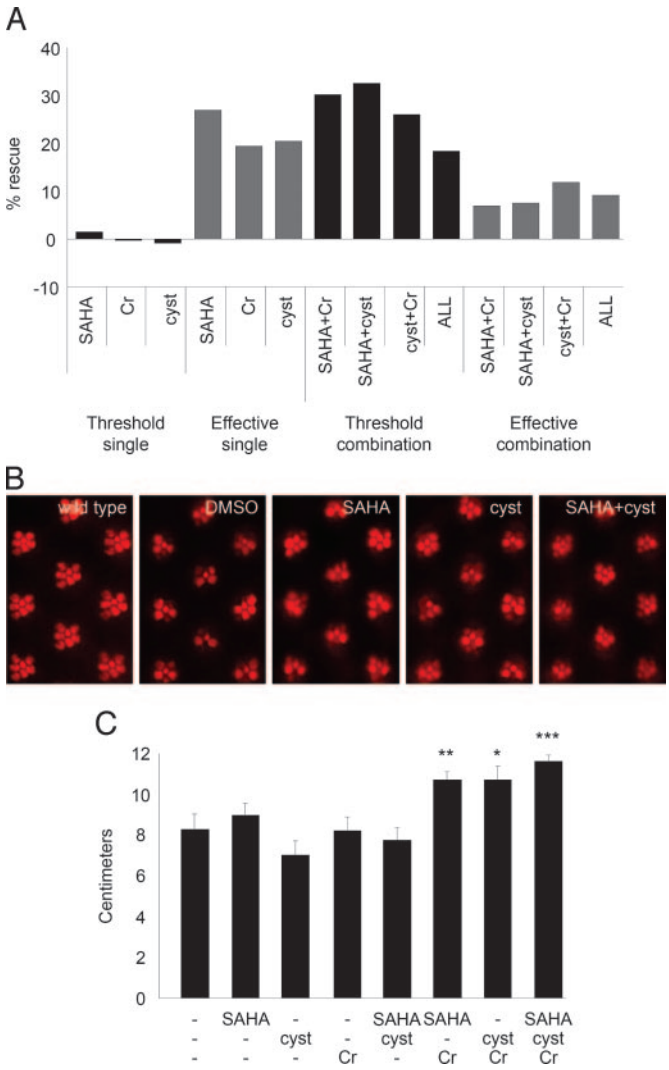

Fig. 3. Combination therapy suppresses photoreceptor degeneration in flies expressing Httex1p. (*A*) Flies expressing Httex1p Q93 were fed threshold or effective concentrations of SAHA, cystamine (cyst), and Congo red (Cr), and their eyes were scored for neurodegeneration at 7 days posteclosion using the pseudopupil technique. Percent rescue was determined as follows: $100 \times (Rt - Rc)/(7 - Rc)$, where *Rt* and *Rc* are rhabdomeres/ommatidium in the treated and control groups, respectively. (*B*) Photographs from ommatidia of 7-day-old flies expressing Httex1p Q93 fed threshold concentrations of SAHA and/or cystamine (cyst) demonstrate the effect observed. (*C*) Combinatorial therapy improves motor function. Climbing ability of adults grown on food supplemented with threshold concentrations of SAHA, cystamine (cyst), and/or Congo red (Cr) in different combinations was evaluated at day 7 posteclosion. For each condition, the average of 20 flies was calculated. ***, $P \ll 0.001$; **, $P < 0.01$; *, $P < 0.05$, compared with DMSO.

effective concentrations, suppression is observed as described previously (16, 22). However, when combinations of SAHA, cystamine, and Congo red are fed to flies at threshold concentrations, degeneration of the photoreceptor neurons is suppressed at levels comparable to or better than the rescue seen with single compound feeding at the effective dose (Fig. 3 *A* and *B*). Flies expressing Httex1p Q93 also show progressively abnormal movements and climbing behavior with age because of impaired motor neuronal function (11, 22). We also evaluated the impact of Congo red, cystamine, and SAHA and their combinations on motor function (Fig. 3*C*). Single threshold doses showed no improvement over DMSO alone. However, in combination, marked improvements in climbing ability and

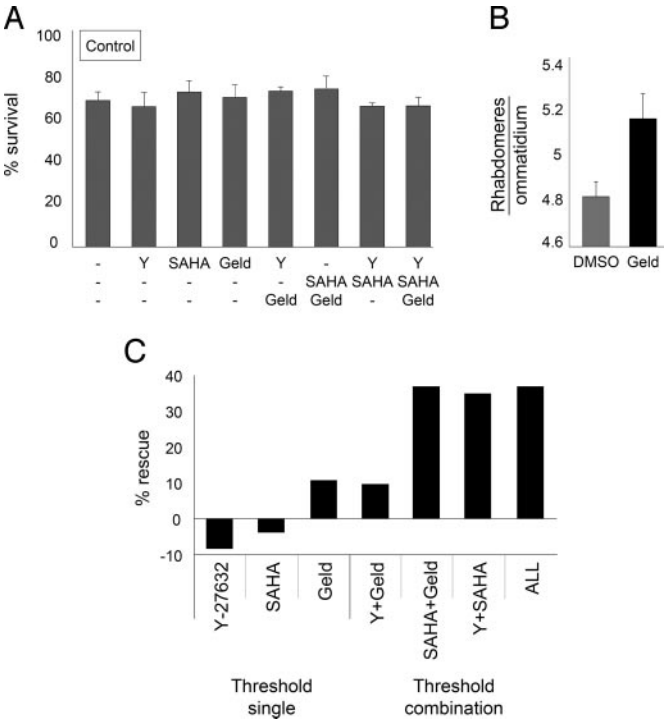

Fig. 4. A distinct regimen of combination therapy suppresses photoreceptor degeneration. (*A*) The effect of compounds singly and in combinations at threshold concentration on flies not expressing Httex1p Q93 showed no change in survival. (*B*) Geldanamycin (Geld) alone at a concentration of 9 μg/ml suppresses photoreceptor neuronal loss. (*C*) Combinations of SAHA with either geldanamycin or Y-27632 (Y) at threshold concentrations effectively reduce neuronal loss. Combinations of geldanamycin and Y-27632 do not show additional suppression of neuronal loss but do not inhibit the ability of other combinations to rescue neurons.

decreased abnormal movements were observed. Thus, in these studies, combinatorial drug therapy at lower doses of drugs proved to be effective at preventing or delaying polyglutamine-induced neuronal loss. In contrast, combinations of drugs at their effective concentrations result in significantly reduced levels of rescue compared to single doses, presumably because of toxic effects of the higher drug loads (Fig. 3*A*).

Drug Combinations Targeting Distinct Cellular Mechanisms. The studies above support the view that targeting distinct cellular mechanisms can potentially result in additive or synergistic relief of pathology. We sought to further test this implication by combining putative therapeutic agents whose mechanism of action is thought to be relatively well understood, namely SAHA, Y-27632, and geldanamycin. SAHA is a histone deacetylase inhibitor that has proven effective at suppressing pathology in both flies (16) and transgenic mouse models of HD (23, 24). The ROCK inhibitor, Y-27632, affects cytoskeletal organization and has been shown to suppress pathogenesis in a *Drosophila* model of HD and aggregate formation in mammalian cells (25). Geldanamycin is an inhibitor of HSP90 that negatively regulates HSP70 levels (26, 27). It has been found to protect against α-synuclein toxicity in a fly model of Parkinson's disease and has been shown to suppress aggregation in a cell culture model of HD (28).

We first explored whether geldanamycin could be effective in suppressing HD pathology in an adult fly. Feeding of geldanamycin alone resulted in amelioration of HD pathogenesis (Fig. 4*B*), demonstrating that geldanamycin is effective in HD as well as Parkinson models.

We next tested whether combinations of these three compounds that target transcription, subcellular organization, and chaperone activity might prove effective at modulating HD pathology. Control experiments demonstrated that at the concentrations used, these drugs exhibited no toxicity in control flies that did not express Httex1p, either singly or in combination (Fig. 4*A*). However, when combinations of these drugs at threshold concentrations that include SAHA with either geldanamycin or Y-27632 were fed to animals expressing Httex1p Q93, degeneration of photoreceptor neurons was strongly suppressed (Fig. 4*C*). On the other hand, combinations that include only geldanamycin and Y-27632 did not lead to additional improvement in degeneration pathology (Fig. 4*C*). Thus, pretesting in *Drosophila* can identify promising pharmacologic combinations and regimens for testing in mammals and can reveal other combinations that either are redundant or for other reasons seem less promising (e.g., geldanamycin with Y-27632).

Discussion

In the quest to find pharmacologic treatments for human diseases, many surrogate markers are used in the screening process, but *in vivo* verification of efficacy remains essential. In addition to demonstrating efficacy or lack thereof, *in vivo* testing employs a system with a level of biological complexity that can often reveal unexpected or untoward effects of various treatment regimens. Transgenic flies engineered to express mutant human genes have been used to test the effectiveness of various chemical compounds *in vivo* (16, 22, 28). Here, we report studies to evaluate the effectiveness of combinatorial drug treatment in suppressing HD pathology. Two combinatorial regimens were evaluated.

The first regimen used Congo red, cystamine, and SAHA in a proof of principle study. Congo red had been found empirically to affect the formation and stability of inclusions. It selectively binds to β-sheet structures (29, 30) and is hypothesized to inhibit formation of potentially toxic polyQ fibrils, possibly by interfering with nucleation events leading to aggregation and/or inhibiting the growth of fibrils (31).

Cystamine was selected based on its ability to inhibit transglutaminase activity and thereby inhibit aggregate formation possibly by blocking glutamine crosslinking (32). It has proven efficacious in prolonging survival and improving motor neuron function in HD mice (32, 33) and reducing photoreceptor neuron degeneration in flies (22, 33). Although its mechanism of action may involve inhibition of transglutaminase activity, other effects that may be relevant have been reported including inhibition of caspase-3, improvement of mitochondrial function, and reduced oxidative stress through the enhanced production of glutathione (34).

Histone deacetylase inhibitors such as SAHA have proven effective at arresting progressive neuronal degeneration in a fly model of HD even after onset of symptoms (16) as well as in transgenic mouse models of HD (23, 24); they are thought to act by improving transcriptional output in an otherwise transcriptionally repressed environment.

We find that combinations of these three drugs at threshold concentrations in *Drosophila* produce no apparent lethality and can suppress polyglutamine-induced pathology at levels comparable to or better than single compound feedings at higher effective concentrations. The results suggest that these three drugs actually target separate aspects of cellular pathology and that the beneficial effects can be additive or synergistic.

The second combinatorial regimen made use of SAHA together with two very differently acting agents, Y-27632 and geldanamycin. Geldanamycin has been shown to relieve Parkinson's-like pathology in an *in vivo* model of flies expressing α-synuclein (28). It is also capable of activating a stress response that inhibits Htt aggregation in mammalian cells (26). We find

that geldanamycin also suppresses HD-like pathology in *Drosophila*. Y-27632 is a well studied inhibitor of the Rho-associated kinase, p160ROCK, that has been found to suppress polyQ aggregate formation in cultured cells and pathology in *Drosophila* (25). These studies implicated Rac1 and more specifically its contribution to the cytoskeleton as important for polyQ pathology.

Combinations that involved SAHA with either geldanamycin or Y-27632 produce a robust suppression of photoreceptor degeneration indicating strong additivity of effects. However, combinations of geldanamycin with Y-27632 exhibit no additional improvement in degeneration phenotype (Fig. 4*C*), suggesting that these two drugs may share common features in their mechanism of action and will not produce a greater relief of pathology when given together.

Combinatorial drug therapies, such as those tested here, may be effective in ameliorating symptoms while reducing side effects in patients with neurodegenerative diseases. Several other compounds, including rapamycin (35) and mithramycin (36), have shown efficacy in *Drosophila* and/or mouse models of HD, and new agents are being identified at a rapid pace, providing additional combinatorial regimens that may be evaluated in future studies. We have explored whether *Drosophila* may provide a cost-effective platform for testing large matrices of drug combinations for optimal combinations of therapeutic drugs, and to test for undesirable interactions, before proceeding to mouse models or patients suffering from HD. For this strategy to be effective, there must be good agreement between pharmacologic responses in flies and mammals. To date, the concordance of therapeutic strategies that behave similarly in flies and mammals has been excellent both in terms of pharmacologic responses in the two systems as well as for different disease models in the two systems (5), and the list continues to grow. Preclinical *in vivo* testing strategies such as those described here could result in a great savings of cost and time in developing potential disease treatments and can serve to identify treatment regimens that are very likely to provide therapeutic benefit to patients.

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