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Chemical enhancement of torsinA function in cell and animal models of torsion dystonia

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SUMMARY

Movement disorders represent a significant societal burden for which therapeutic options are limited and focused on treating disease symptomality. Early-onset torsion dystonia (EOTD) is one such disorder characterized by sustained and involuntary muscle contractions that frequently cause repetitive movements or abnormal postures. Transmitted in an autosomal dominant manner with reduced penetrance, EOTD is caused in most cases by the deletion of a glutamic acid (ΔE) in the *DYT1* (also known as *TOR1A*) gene product, torsinA. Although some patients respond well to anticholingerics, therapy is primarily limited to either neurosurgery or chemodenervation. As mutant torsinA (ΔE) expression results in decreased torsinA function, therapeutic strategies directed toward enhancement of wild-type (WT) torsinA activity in patients who are heterozygous for mutant *DYT1* may restore normal cellular functionality. Here, we report results from the first-ever screen for candidate small molecule therapeutics for EOTD, using multiple activity-based readouts for torsinA function in *Caenorhabditis elegans*, subsequent validation in human *DYT1* patient fibroblasts, and behavioral rescue in a mouse model of *DYT1* dystonia. We exploited the nematode to rapidly discern chemical effectors of torsinA and identified two classes of antibiotics, quinolones and aminopenicillins, which enhance WT torsinA activity in two separate in vivo assays. Representative molecules were assayed in EOTD patient fibroblasts for improvements in torsinA-dependent secretory function, which was improved significantly by ampicillin. Furthermore, a behavioral defect associated with an EOTD mouse knock-in model was also rescued following administration of ampicillin. These combined data indicate that specific small molecules that enhance torsinA activity represent a promising new approach toward therapeutic development for EOTD, and potentially for other diseases involving the processing of mutant proteins.

INTRODUCTION

Dystonia is a movement disorder characterized by involuntary twisting and contraction of skeletal muscles. Collectively, different types of dystonia are estimated to affect over 3 million people worldwide (Defazio, 2007). Early-onset torsion dystonia (EOTD) is a severe and heritable form of dystonia that is transmitted in an autosomal dominant manner with reduced penetrance (30-40%). This disorder is associated with mutations in the human *DYT1* gene (also known as *TOR1A*) that result in the loss of one of a pair of glutamic acid residues (ΔE302/303) in the C-terminal region of a protein termed torsinA (Ozelius et al., 1997). Torsins (a collective term for torsinA and its homologs) are members of the large and functionally diverse AAA+ (ATPases associated with cellular activity) family of ATPase proteins. Several AAA+ proteins function as oligomeric complexes involved in facilitating protein folding and guidance to cellular locations (Hanson and Whitehouse, 2005). In this regard, torsinA is predicted to affect the conformational state of proteins and their interactions with other proteins (Goodchild et al., 2005; Kock et al., 2006).

Consistent with a role in protein folding or trafficking, evidence from multiple labs and systems indicates that the endoplasmic reticulum (ER) is a predominant site of torsin localization (Hewett et al., 2000; Kustedjo et al., 2000; Basham and Rose, 2001; Liu et al., 2003; Goodchild et al., 2005; Callan et al., 2007). Indeed, torsins exhibit chaperone-like activity in vivo and have been shown to prevent the accumulation of both α-synuclein and polyglutamine-repeat-containing fusion proteins when overexpressed (McClean et al., 2002; Caldwell et al., 2003). More recently, functional analyses of endogenous torsinA activity in human fibroblasts indicate that it is involved in the trafficking of proteins through the ER secretory pathway, with loss/downregulation of torsinA, or mutant torsinA, interfering with this process (Hewett et al., 2007; Hewett et al., 2008). It was also demonstrated recently that mutations in the ε-sarcoglycan gene (*DYT11*, also known as *SGCE*) product (SGCE), which are associated with myoclonus dystonia, result in an ER-based trafficking defect, and that overexpressed mutant SGCE proteins were selectively degraded in the presence of torsinA (Esapa et al., 2007). These combined studies strongly suggest that a normal activity of torsinA involves maintaining the homeostatic balance of proper protein folding and processing in the cell, and that the loss of torsinA function in dystonia potentially renders cells more susceptible to intracellular stressors, such as those associated with the unfolded protein response (UPR) (Hewett et al., 2003; Cao et al., 2005; Zhang and Kaufman, 2006).

Mutant torsinA (ΔE) exhibits reduced ATPase activity as compared with WT torsinA, and the mutant form inhibits WT ATPase activity in mixed protein preparations in vitro (Pham et al., 2006). It has been further demonstrated, in cell culture, that the absence of the glutamic acid (ΔE) residue in torsinA can result in the formation of aberrant membranous inclusions and increased subcellular distribution of this protein at the nuclear envelope (NE)

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(Gonzalez-Alegre and Paulson, 2004; Goodchild and Dauer, 2004; Naismith et al., 2004). Other studies support a role for torsinA in cytoskeletal dynamics interactions through its interaction with nesprins and their associated protein partners in the lumen of the NE, affecting the role of these proteins in nuclear and cell movement (Nery et al., 2008). Similarly, mutations in an ER-resident, embryonically expressed *C. elegans* torsinA ortholog, OOC-5, cause deficits in nuclear rotation (Basham and Rose, 2001). These reports imply that the molecular nature of this disease might be a combined consequence of reduced torsinA activity causing ER and NE dysfunction. Although studies pertaining to mutant torsinA (ΔE) localization are potentially informative with respect to the molecular pathology of EOTD, they are not readily amenable to drug-screening approaches, which depend on quantitative profiling of drug potency or efficacy.

In contrast to other movement disorders, such as Parkinson's disease, where large populations of neurons are lost, there is no obvious neurodegeneration in EOTD patient brains (Rostasy et al., 2003; McNaught et al., 2004). Therefore, subtle causes of cellular dysfunction are most likely to be responsible for the symptomatic features of dystonia, possibly related to defects in secretion that compromise neurotransmission, or to increased sensitivity to ER stress, which compromises neuronal integrity. Considering this, along with the reduced penetrance associated with mutation in the human *DYT1* gene, EOTD represents an outstanding candidate disorder for therapeutic intervention, since even incremental increases in torsinA activity could potentially restore normal movement control to the dysfunctional neuronal circuitry.

Here, we take advantage of functional assays for torsinA activity in the nematode roundworm, *C. elegans*, to rapidly screen through FDA (U.S. Food and Drug Administration)-approved molecules for those that enhance the chaperone activity associated with this protein in vivo. *C. elegans* is an organism that can be cultured rapidly with an experimentally tenable life span of around 17 days, in which exploratory concepts can be taken to mechanistic fruition quickly and inexpensively. Furthermore, this animal has a transparent anatomy, allowing for transgenic green fluorescent protein (GFP) markers to be followed over the course of development and aging. The recent application of *C. elegans* toward human disease research has already provided insights into the function of specific gene products linked to a variety of neurological disorders (Faber et al., 2002; Caldwell et al., 2003; Williams et al., 2004; Parker et al., 2005; Hamamichi et al., 2008; Caldwell and Caldwell, 2008; Gitler et al., 2009). Furthermore, worms are now being exploited as a system for therapeutic drug screening (reviewed in Kaletta and Hengartner, 2006).

Following successful drug screening for modifiers of torsinA activity using transgenic *C. elegans*, we identified specific molecules in two drug classes that enhanced the activity of wild-type torsinA. Since it is not realistic that all functional features of torsinA activity can be recapitulated in one simple animal model, we examined the efficacy of candidate drugs to reverse a phenotype associated with human *DYT1* patient fibroblasts. These patient cells exhibit a defect in the processing of a naturally secreted luciferase that appears to reflect reduced function of torsinA as an ER-resident protein (Hewett et al., 2007). Thus, this assay represents an independent mechanism for examining the efficacy of torsinA-dependent drugs in the endogenous cellular context. Notably, one positive drug candidate emerging from this assay was evaluated further in a mouse model of human *DYT1* dystonia (Dang et al., 2005). The mice in this model were engineered to genetically mimic human *DYT1* dystonic patients; that is, they were heterozygous and had one *Dyt1* wild-type allele and one mutant torsinA (ΔE) allele, and as a result, the male animals had a significant behavioral movement defect in beam walking (Dang et al., 2005). We discovered that this behavioral deficit was rescued with the administration of a positive drug candidate – an antibiotic, ampicillin. Therefore, a drug that was first identified through a medium-throughput *C. elegans* screen, and verified in human patient cells, restored function to a mouse knock-in model of this disease, thus providing essential validation of ampicillin as a therapeutic lead for EOTD within the context of the complex physiology of a whole mammalian system.

RESULTS

Chemical screening for small molecule effectors of torsinA chaperone activity

As a primary strategy toward the identification of chemical modifiers of torsinA, we utilized an assay whereby quantitative changes in the previously demonstrated molecular chaperone activity of this protein could be readily evaluated. By fusing varying lengths of polyglutamine repeats to GFP, protein misfolding can be induced (Satyal et al., 2000; Caldwell et al., 2003). Although GFP with 19 glutamine residues (Q19) appears soluble and diffuse, expanded glutamine repeats (Q82) cause the fusion protein to misfold and form aggregates when expressed in the body wall muscles of *C. elegans* (Fig. 1A,B, triangle). Ectopic overproduction of human wild-type torsinA (WT) reduces the formation of Q82 aggregates (Fig. 1A,B, diamond), but this chaperone activity is abolished when EOTD-associated mutant torsinA (ΔE) is co-expressed with the Q82 fusion protein (Fig. 1A,B, circle). Notably, this ΔE mutation appears to induce an overall loss-of-torsinA function, as co-expression of WT and mutant (ΔE) torsinA together does not yield intermediate chaperone activity, but instead exhibits levels similar to that of mutant torsinA (ΔE) alone (Fig. 1A,B, square), thereby recapitulating the dominant nature of the disease state.

These isogenic, transgenic nematode strains were used to quantitatively measure changes in protein misfolding as a functional readout in a screen for small molecule effectors of torsinA activity. We screened the Prestwick *C. elegans* library, a collection of 240 chemically and therapeutically diverse drugs (pre-screened for maximal dosage and toxicity to worms), for compounds that either inhibited the dominant effect of mutant torsinA (ΔE) or that enhanced the normal activity of WT torsinA (supplementary material Fig. S1A). This screen was performed in worms that presumably best reflected the human *DYT1* genetic condition, in that they expressed both the WT and ΔE gene products (WT/ΔE). At the third larval stage (L3), a 60% increase in aggregates was reproducibly observed in worms expressing WT/ΔE torsinA when compared with animals expressing only the WT human protein (Fig. 1B). This differential represented an ideal developmental stage for readily distinguishing molecules that significantly altered torsinA chaperone function.

A primary screen revealed seven molecules that significantly decreased protein aggregation in worms expressing WT/ΔE torsinA

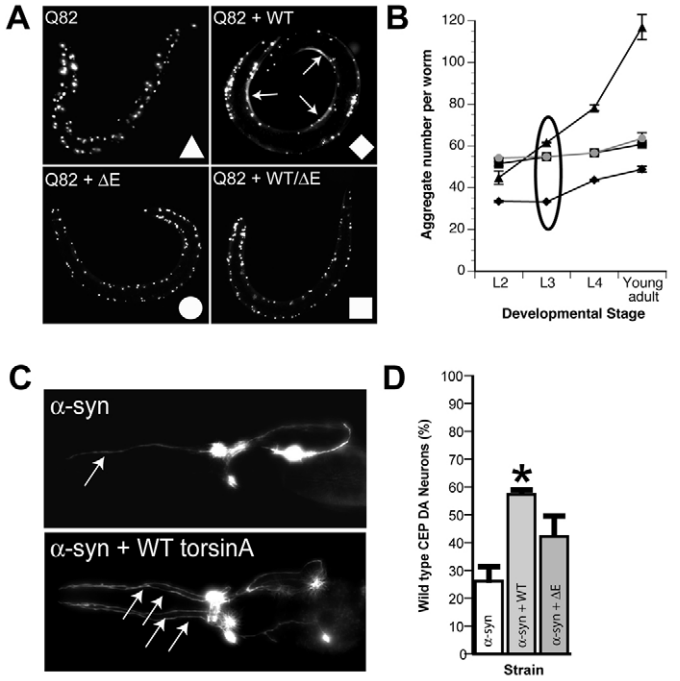

Fig. 1. Assays for torsinA activity used in drug screening procedures. (A,B) Human torsinA (WT) suppresses Q82::GFP (Q82) aggregation in the body wall muscle cells of *C. elegans*. (A) Representative images of the following worm strains at the L3 stage: $P_{unc\text{-}54}$::Q82::GFP (Q82), $P_{unc\text{-}54}$::Q82::GFP + $P_{unc\text{-}54}$::torsinA (Q82 + WT), $P_{unc\text{-}54}$::Q82::GFP + $P_{unc\text{-}54}$::torsinA(ΔE) (Q82 + ΔE), and $P_{unc\text{-}54}$::Q82::GFP + $P_{unc\text{-}54}$::torsinA + $P_{unc\text{-}54}$::torsinA(ΔE) (Q82 + WT/ΔE). Arrows indicate soluble GFP and symbols represent the worm strains depicted in B. (B) The average aggregate number per worm at different developmental stages (larval stages L2-L4 and young adults) of Q82 (triangle), Q82 + WT (diamond), Q82 + ΔE (circle) and Q82 + WT/ΔE (square) worms. Data points are the mean aggregate number of 30 worms for each transgenic line at each time point. The oval indicates the stage used to examine compound exposure of Q82 + WT/ΔE worms. (C,D) torsinA prevents dopamine neuron (DA neuron) neurodegeneration resulting from the overexpression of α-syn in *C. elegans* as animals age. (C) Representative images of a worm displaying DA neuron degeneration [top image, $P_{dat\text{-}1}$::GFP + $P_{dat\text{-}1}$::α-syn (α-syn)], in which only one cephalic (CEP)-class DA neuron is intact (arrow), and a torsinA-rescued worm [bottom image, $P_{dat\text{-}1}$::GFP + $P_{dat\text{-}1}$::α-syn + $P_{dat\text{-}1}$::torsinA (α-syn + WT)], in which all four of the CEP-class neurons (arrows) are protected. (D) Quantitative analysis of the average CEP DA neuron degeneration in isogenic lines of worms expressing α-syn, α-syn + WT, or α-syn + ΔE at the 4-day-old adult stage. Data points are the means of three independent experiments ± the standard error of the mean (S.E.M.); **P*<0.05.

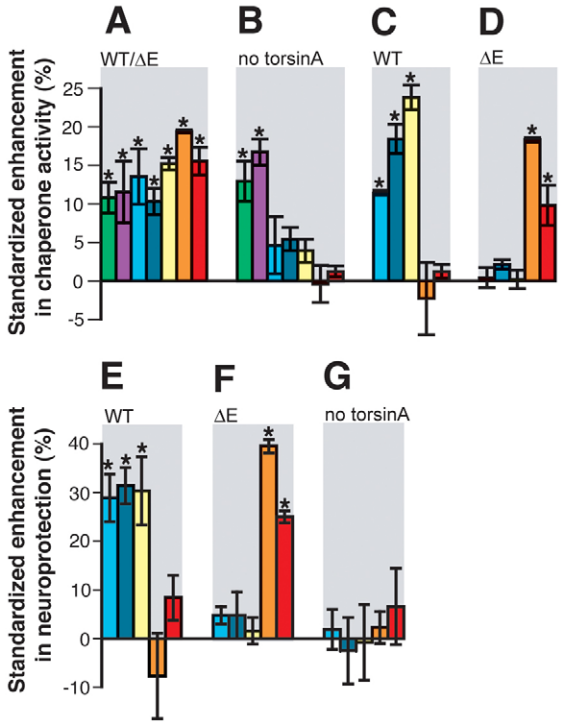

Fig. 2. Analysis of positive drug candidates in different transgenic worm backgrounds. (A-D) Drug efficacy is depicted for color-coded compounds as the standardized enhancement in chaperone activity for torsinA. The values were calculated using the following formula: (A – B)/A, where 'A' stands for the average aggregate number in the solvent control and 'B' represents the average aggregate number in the treatment. Data points are the mean from three independent experiments (with 30 L3-stage worms/analysis) ± S.E.M.; the statistical significance compared with respective solvent controls in A and with treatment in B was calculated by *t* test, **P*<0.05. The genetic backgrounds analyzed were as follows: (A) Q82 + WT/ΔE, (B) Q82 alone (no torsinA), (C) Q82 + WT and (D) Q82 + ΔE. (E-G) torsinA dependency of the five compounds that were identified in the initial polyglutamine screen for DA neuroprotection. The standardized enhancement in DA neuroprotection for torsinA for each compound was analyzed in 4-day-old adult worms with the following genetic backgrounds: (E) α-syn + WT, (F) α-syn + ΔE, and (G) α-syn alone (no torsinA). Data points are the mean of 35-70 worms for each genetic background from three independent experiments ± S.E.M. Each value was compared with its appropriate vehicle control and statistical significance calculated by *t* test, **P*<0.05.

(Fig. 2A). Since these drugs could potentially target Q82-induced GFP aggregates directly, without modulating torsinA activity, we exposed worms lacking torsinA expression (Q82 alone) to these compounds and found that two of the drugs, mafenide hydrochloride (green) and dyclonine hydrochloride (purple), significantly reduced protein aggregation in the absence of torsinA (Fig. 2B). Having eliminated these non-specific candidates, we subsequently exposed distinct populations of Q82 animals, expressing either WT or mutant (ΔE) torsinA, to the remaining five drugs (Fig. 2C,D), and found three compounds [two quinolones, nalidixic acid (turquoise) and oxolinic acid (blue), as well as a β-lactam antibiotic, metampicillin (yellow)] that exclusively enhanced WT torsinA activity (Fig. 2C). Two other drugs, an L-type Ca^{2+} channel antagonist, loperamide hydrochloride (orange), and a cyclooxygenase (COX) inhibitor, meclofenamic acid (red), selectively neutralized mutant torsinA (ΔE) inhibitory activity (Fig. 2D).

Secondary testing of compounds by analysis of torsinA activity in neuroprotection

We previously reported that torsinA protects *C. elegans* dopamine neurons (DA neurons) from age-dependent neurodegeneration induced by the overexpression of human α-synuclein (α-syn), a protein directly associated with familial Parkinson's disease (Fig. 1C,D) (Singleton et al., 2003; Cao et al., 2005). As a secondary screen, we tested the five positive compounds from our initial

screen for torsinA-dependent neuroprotection in α-syn worms (Fig. 2E-G; supplementary material Fig. S1B). The quinolones and β-lactam molecules exhibited a protective effect on animals expressing only WT torsinA (Fig. 2E), whereas the Ca^{2+} channel antagonist and COX inhibitor resulted in neuroprotection only in the presence of mutant torsinA (ΔE) (Fig. 2F). Importantly, none of these compounds enhanced DA neuron survival from α-syn overexpression in a genetic background where neither WT torsinA nor torsinA (ΔE) was present (Fig. 2G). Thus, these five compounds displayed reproducible changes in activity and specificity for either WT or mutant (ΔE) torsinA in two diverse nematode models, which included separate cell types (muscle vs neuron) with torsinA variants expressed from different promoters (P_{unc-54} vs P_{dat-1}) and with distinct functional readouts (polyQ-induced misfolding vs α-syn neurotoxicity).

Structure-activity relationships between torsinA effector molecules

The molecular etiology of EOTD is believed to result from a dominant negative loss of torsinA activity mediated by the mutant protein (Pham et al., 2006); thus, molecules that function to elevate the activity of the remaining WT torsinA within patients may represent the best candidates for therapy (i.e. quinolone and β-lactam antibiotics). An examination of 'cousin' molecules in the quinolone and β-lactam classes was performed in worms expressing Q82 + WT torsinA to determine whether they could enhance WT torsinA activity. Including the originally identified molecules, five out of seven quinolones and seven out of ten β-lactam antibiotics significantly enhanced torsinA activity in transgenic WT/ΔE worms expressing Q82::GFP (supplementary material Figs S2 and S3). Using these data, gross structure-activity relationship (SAR) modeling was undertaken.

Both antibiotic classes appear to have three general components (Fig. 3A,B) that surround antibiotic cores [quinolones contain a bicyclic core (Fig. 3A, red) and aminopenicillins have a β-lactam nucleus (Fig. 3B, red)]. The common chemical moieties include: (1) a region of alternating H-bond donor and acceptor (blue); (2) a variable hydrophobic region (gray); and (3) a permissive region with a high degree of variability (green). Following an alignment of structures between quinolones and aminopenicillins using representative molecules, the relative positioning of chemical functionality can be observed (Fig. 3C,D). Furthermore, a pharmacophoric model was constructed by aligning the structures of five quinolones and three aminopenicillins that enhanced WT torsinA activity (Fig. 3E). These SAR data imply that there is some structural relatedness between the two classes of antibiotics that enhance WT torsinA activity. Additionally, these data suggest that the antibiotic core regions of the quinolones and aminopenicillins are simply scaffolds that present the appropriate pharmacophoric elements (i.e. the alternating H-bond donor/acceptor) (Fig. 3E, arrow).

Target validation in human EOTD patient fibroblasts

To validate the primary outcomes of our screening efforts, we investigated the efficacy of representative and commonly prescribed drugs from these two classes in human EOTD patient cells. Fibroblasts from affected *DYT1* patients (WT/ΔE at endogenous levels) do not process proteins through the endoplasmic reticulum

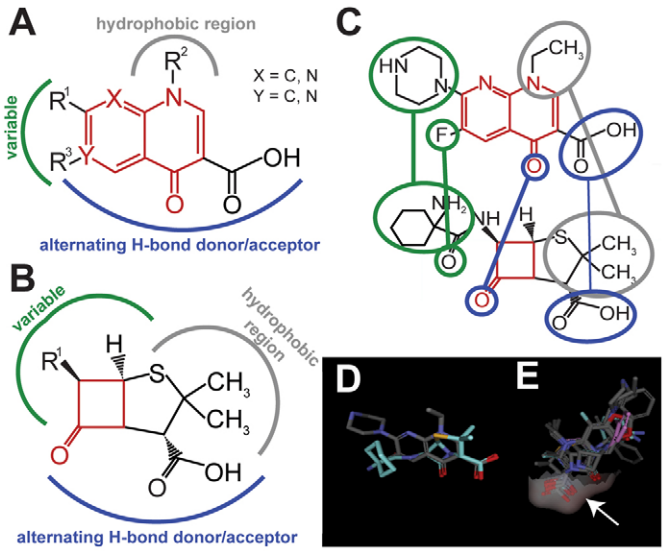

Fig. 3. SAR modeling of quinolone and β-lactam antibiotics reveals structural relatedness. (A) The structure of a generalized quinolone showing the bicyclic antibiotic core (red) and surrounding chemical moieties. (B) The structure of an aminopenicillin antibiotic showing the β-lactam nucleus (red) and surrounding chemical moieties. (C) Alignment of structures between a quinolone (norfloxacin; top structure) and an aminopenicillin (cyclacillin; bottom structure) displaying the relative chemical positions of the common components. (D) Alignment of norfloxacin (gray) and cyclacillin (light blue). (E) A pharmacophoric model using alignment of the structures of five quinolones and three aminopenicillins that enhanced WT torsinA activity. The antibiotic core region of the active molecules may function as a scaffold for presenting the alternating H-bond donor/acceptor, which is shown in red (arrow).

(ER) as efficiently as control fibroblasts (WT), as demonstrated by markedly less secretion of Gaussia luciferase (Gluc) activity into the media (Badr et al., 2007; Hewett et al., 2007). We examined the secretion of Gluc in *DYT1* and control cells, with and without antibiotic exposure. One quinolone, enoxacin, significantly impaired secretion in both cell types (supplementary material Fig. S4). By contrast, ampicillin significantly enhanced secretion in both control (P=0.0097) and EOTD patient (P=0.0039) cells (Fig. 4A,B). Furthermore, a concentration of between 3 and 6 μg/ml of ampicillin was sufficient to restore the Gluc secretion of patient fibroblasts back to the level of control fibroblasts.

Functional validation by behavioral analysis in a torsinA dystonic mouse model

Although the nematode and human patient cell data that were obtained strongly support the impact of ampicillin as a functional modifier of torsinA activity, the translational value of these findings would be further augmented by evaluation in a rodent model of dystonia. Therefore, as further functional validation of these results, we tested whether ampicillin can reverse a behavioral defect associated with mutant torsinA in an established and well-characterized knock-in mouse model of EOTD that genetically mimics the human condition (Fig. 4C,D) (Dang et al., 2005). Heterozygous *Dyt1* ΔE knock-in mice exhibit significant deficits in the beam-walking test, a measure of fine motor coordination and

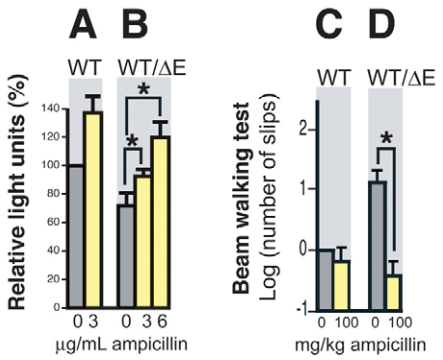

Fig. 4. Ampicillin restores normal torsinA readouts in EOTD patient cells and in a mouse knock-in model for EOTD. (A,B) Control (WT) and *DYT1* (WT/ΔE) fibroblasts were infected with a vector encoding Gluc and, 72 hours later, treated with saline as a vehicle control (gray bars) or ampicillin (yellow). Gluc secretion was then measured over the following 48-hour period. Light unit values were standardized to 100% for control cells treated with vehicle only. Both 3 and 6 µg/ml of ampicillin restored *DYT1* fibroblast secretion to levels that were not significantly different from control cells treated with vehicle. (C,D) Ampicillin treatment significantly improved motor coordination and balance in *Dyt1* ΔGAG knock-in mice (WT/ΔE). Following administration of a second intraperitoneal injection of ampicillin (day 4), ΔE knock-in mice showed significantly fewer slips and an improvement in beam-walking tests. Data were normalized to control mice that were injected with vehicle (saline) solution. *P<0.05. Vertical bars represent mean ± S.E.M.

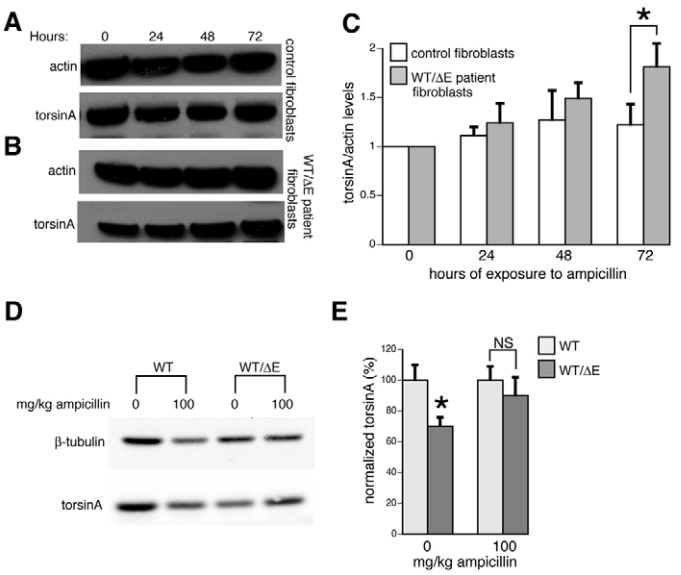

Fig. 5. Effect of ampicillin treatment on the levels of torsinA in human *DYT1* (WT/ E) patient fibroblasts and in *Dyt1* GAG (WT/ E) knock-in mouse striatum. (A,B) Representative blots from one of the four different experiments carried out using the control fibroblast line (HF71) (A), and from one of the three different experiments using a *DYT1* patient fibroblast line (HF49) (B), treated with ampicillin over the course of 72 hours. Western blotting was performed using anti-β-actin and anti-torsinA antibodies. (C) Densitometry was performed to quantitate torsinA and actin bands on all western blots. The average of the ratio of torsinA and actin levels is expressed as the mean ± S.D. for all time points. The asterisk represents a significant difference in torsinA levels in *DYT1* (WT/ΔE) fibroblasts compared with the control (WT) fibroblast line after 72 hours of treatment with ampicillin (P<0.0015). (D) Representative images of the western detection for β-tubulin and torsinA in extracts of treated WT or knock-in (WT/ΔE) mouse striatum. Genotypes [control littermate (WT) and knock-in (WT/ΔE)] and the treatments (0 or 100 mg/kg ampicillin) of the samples are shown. (E) Quantified torsinA level in the striatum. TorsinA levels were normalized to β-tubulin and each group was statistically compared with the level in the vehicle-treated WT mice. The vertical bars represent mean ± S.E.M. *P<0.05.

balance. Over multiple trials, ΔE knock-in mice that were injected with vehicle alone showed more beam slips on average compared with control mice. However, ΔE knock-in mice treated with ampicillin displayed a significant reduction in the number of slips (P=0.03), such that ΔE knock-in mice treated with vehicle (saline) showed about three times as many slips as the ampicillin-treated group. In control mice, no significant difference between the vehicle and the ampicillin-treated groups was observed (P=0.85). Importantly, there was no significant difference between ampicillin-treated knock-in mice and control mice (either for control mice treated with vehicle solution, P=0.47, or for control mice treated with ampicillin, P=0.51), suggesting that the motor performance in torsinA ΔE knock-in mice was restored to the same level as in controls (Fig. 4D).

Mechanistic studies

The genomes of *C. elegans*, mice and humans do not have homologs of bacterial transpeptidases, the established targets of β-lactam antibiotics. Thus, we conclude that ampicillin must be exerting an effect on torsinA through a novel mechanism. Since WT torsinA is localized to the ER lumen, one possibility is that ampicillin is impacting the unfolded protein response (UPR), a cellular reaction to protein misfolding stress (Zhang and Kaufman, 2006). We therefore assessed this possibility by exposing worms to tunicamycin, an inducer of ER stress, and treated these animals with ampicillin. Using an established UPR reporter, *hsp-4*::GFP (Calfon et al., 2002), in the worm intestine, where the cells lack endogenous *C. elegans* torsin protein expression (Cao et al., 2005), animals were evaluated for any change in stress response. At the concentrations of drug that were previously used for enhancement of torsinA activity, no significant difference was observed, as exhibited by robust *hsp-4*::GFP expression (supplementary material Fig. S5A). Thus, it is apparent that the activity that we observed in facilitating the processing of reporter and misfolded proteins among our screened target cells was not the result of a torsinA-independent effect on ER stress through the UPR.

Our data, indicating that enhancement of torsinA activity by ampicillin results in a significant increase in neuroprotection, are reminiscent of a previous report where it was shown that specific β-lactam antibiotics function in a neuroprotective capacity through potent stimulation of glutamate transporter 1 (GLUT1) transcription in cultured cells (Rothstein et al., 2005). As the functional assays that we used to identify and characterize torsinA-dependent small molecule effectors in *C. elegans* were conducted in cell types lacking this transporter, it is unlikely that this same mechanism of action is responsible for the change in activity that we observed. Nevertheless, to rule out any potential change or ectopic expression of GLUT1, we undertook an analysis of worms expressing a characterized nematode GLUT1 reporter, *glt-1*::GFP

(Mano et al., 2007). As expected, following exposure to ampicillin, we did not observe enhanced *glt-1*::GFP expression in transgenic animals when compared with vehicle control; these data were analyzed by scoring fluorescence pixel intensity and by semi-quantitative reverse transcription (RT)-PCR of *glt-1* mRNA levels (supplementary material Fig. S5B,C). These data suggest that the neuroprotective enhancement of torsinA activity that we observed in response to small molecule treatment appears to be independent of GLUT1 expression in *C. elegans*.

Molecular chaperones can either inhibit initial protein misfolding or resolve existing aggregates (Mogk et al., 1999). Since, in our initial worm screen, both torsinA and Q82 were expressed simultaneously from the same promoter, distinguishing between these scenarios was precluded. Metampicillin, which specifically enhances WT torsinA, provided an opportunity to investigate torsinA chaperone activity on pre-existing aggregates in L2-stage Q82 + WT/ΔE worms. Treated animals exhibited a significant decrease in pre-formed Q82 aggregates commensurate with a limited drug exposure regimen, suggesting that torsinA has the capacity to not only prevent misfolding, but also to disaggregate existing protein aggregates (supplementary material Fig. S5D,E).

To explore the prospect that the observed enhancement of torsinA activity is a consequence of altered protein levels, we evaluated ampicillin-treated human fibroblasts from *DYT1* dystonia patients by western blotting. Fibroblasts from a control fibroblast line (HF71) and a *DYT1* patient (HF49) were treated (or not) with ampicillin over a course of 72 hours. The results of this analysis showed a gradual increase of torsinA levels in the patient cells over time, with little to no effect in control cells (Fig. 5A-C). After 72 hours, there was a significant increase in torsinA levels in *DYT1* fibroblasts when compared with the control fibroblasts ($P<0.0015$). These data indicate that ampicillin treatment of human *DYT1* patient fibroblasts in the endogenous heterozygous disease condition (WT/ΔE) resulted in an increase in total torsinA protein levels. These data correlate with our findings that also show that there is a significant increase in secretion from patient fibroblasts after 72 hours of treatment with ampicillin (Fig. 4A,B). Previous work has shown that, when torsinA expression was decreased in control fibroblasts by small interfering RNAs (siRNAs), the levels of Gluc secretion were decreased by 51% (Hewett et al., 2008). By contrast, when torsinA (ΔE) levels were decreased selectively in *DYT1* patient cells, the levels of Gluc secretion were increased by 42%. Together, these studies suggest that ampicillin acts to increase levels of torsinA in *DYT1* fibroblasts and that this may account for the enhanced secretion of Gluc observed in ampicillin-treated *DYT1* cells.

As further evaluation of the effect of ampicillin treatment on torsinA levels, we examined the striatal brain regions of the *Dyt1* knock-in mice that were used in the behavioral assays. TorsinA levels were compared by western blotting (Fig. 5D,E) following treatment with either vehicle or ampicillin. The results of this analysis indicate that the striatal torsinA level from the dissected brains of knock-in (WT/ΔE) mice was significantly lower than that in the control (WT) mice (70.7%; $P=0.03$) without ampicillin treatment (vehicle only). However, the striatal torsinA levels in the ampicillin-treated WT/ΔE and WT mice were the same as those in the vehicle-treated WT mice ($P=0.660$ and $P=0.894$, respectively). This suggests that the levels of torsinA in the heterozygous animals might be stabilized in the presence of ampicillin.

Although previous reports have indicated a transcriptional effect of β-lactam antibiotics (Rothstein et al., 2005; Schumann et al., 2005; Lee et al., 2008), it is unlikely that this represents an explanation for the observed enhancement of torsinA activity in all of our studies. First, the nematode screens that identified the enhanced activity were conducted with human torsinA variants that were ectopically expressed from distinct promoters in two different cell types: body wall muscles and DA neurons (Fig. 1). Likewise, in both the human fibroblast and mouse models, drug treatment did not result in an observable increase in torsinA protein level in the WT backgrounds, thereby arguing against a generalized transcriptional effect. It remains to be fully discerned, however, whether the enhancement of torsinA activity is due to increased synthesis or stabilization, or decreased degradation, of torsinA, and whether there is a differential effect on mutant versus WT torsinA.

DISCUSSION

Successful efforts at drug discovery are dependent upon the experimental capacity to quantitatively evaluate the phenotypic effects of chemical modifiers. Here, we have exploited a combination of functional assays that enable an assessment of the changes to torsinA activity in diverse cellular and animal models. The identification of specific classes of small molecules that reproducibly affect torsinA across multiple activity-based readouts represents an important step in the development of drugs for dystonia therapy. Pharmacological correction of the phenotypic manifestations of EOTD could involve either inhibition of mutant torsinA (ΔE) dysfunction or enhancement of normal WT activity. Promising studies using RNA interference (RNAi) have demonstrated remarkable allele-specific silencing in the knockdown of torsinA (ΔE) to reverse the dominant negative effect of this mutant protein on secretion (Hewett et al., 2008), and to restore the normal cellular distribution of WT torsinA (Gonzalez-Alegre et al., 2005). This existing methodology has utilized either transfection of cholesterol-conjugated siRNAs or lentiviral-mediated delivery of short-hairpin RNAs (shRNAs) to target mutant torsinA (ΔE) transcripts in cell cultures (Gonzalez-Alegre et al., 2005). Although the prospect of RNAi-based therapies is very exciting, such approaches are still under development and face challenges in delineating successful in vivo delivery paradigms. Moreover, the risk associated with the application of such novel technology for a non-degenerative disorder, such as dystonia, will undoubtedly face serious regulatory hurdles prior to the onset of human trials. It also needs to be determined whether, in cells that have been targeted for mutant torsinA (ΔE) knockdown by RNAi, the activity of the one remaining allele for WT torsinA will be sufficient for normal function. It may be that a combinational approach involving RNAi knockdown of mutant torsinA (ΔE), in conjunction with small molecule enhancement of WT torsinA activity, will be required to achieve a successful therapeutic response in patients.

Given that there is only a single amino acid difference between the WT and mutant (ΔE) torsinA proteins, the observed selectivity of the identified molecules indicates that at least a subset of these compounds may act indirectly to affect torsinA function. In this context, it has been suggested that the increased localization of mutant torsinA (ΔE) to the NE reflects aberrant interactions with

different substrates in the NE, which are distinct from other WT torsinA partners at the ER (Bragg et al., 2004; Goodchild and Dauer, 2004; Naismith et al., 2004). Alternately, the targets may not be different per se, but rather, the mutant form may interact differentially with a particular shared substrate. Thus, the unexpected specificity of the compounds revealed through our screen is probably representative of preferential interactions with molecular targets by WT and mutant (ΔE) torsinA variants themselves.

The proclivity of AAA+ proteins, such as torsinA, to function in multimeric complexes suggests that factors influencing the formation and stability of these complexes may impact overall activity. Recent insights from human genetic studies have implicated a single nucleotide polymorphism (SNP), which changes residue 216 of torsinA from an aspartic acid (D) to a histidine (H), as having an impact on the penetrance of *DYT1* dystonia (Kock et al., 2006; Risch et al., 2007). The frequency of the 216H WT allele is increased in non-manifesting torsinA (ΔE) carriers and is decreased in symptomatic dystonia patients. Therefore, the 216H allele is protective in trans, but apparently not in cis, with the (ΔE) mutation. These striking data point to an intermolecular relationship, in which subtle changes in torsinA complexes potentially play a determinative role in the disease state. It should be noted, however, that one study of French dystonia patient populations did not reveal a modifying effect of this allele (Frederic et al., 2009). Other recent reports indicate that SNPs in the 3′-UTR of the *DYT1* gene impact penetrance in EOTD and are suggestive of an association with focal forms of late-onset dystonia (Clarimon et al., 2005; Kamm et al., 2006; Risch et al., 2007). The evidence for cis regulatory SNPs having functional consequences is further indicative of the importance of maintaining a proper intracellular balance of torsinA levels. In this regard, it has been shown recently in *Dyt1* mouse models that very high levels of either mutant or WT torsinA yield phenotypically similar defects (Grundmann et al., 2007). Further studies involving the small molecule effectors that we have identified as enhancers of torsinA activity may reveal a role for these compounds in stabilizing or increasing the function of torsinA complexes influenced by these polymorphisms.

The identification of ampicillin as a potent enhancer of torsinA activity in worms, mutant mice and human *DYT1* patient cells is cause for significant hope that novel therapeutic approaches to dystonia may be on the horizon. However, cautionary measures are required to restrict the unbridled use of the identified antibiotics for this potential application, as this could result in superinfections and death, as well as drug-resistant bacteria. This warrants continued efforts to evaluate additional aminopenicillin-like molecules, which function poorly as antibiotics, yet still enhance torsinA activity in vivo, as candidates for long-term EOTD therapy. Nevertheless, our current results represent a valuable lead for subsequent medicinal chemistry experiments aimed at discerning an optimal drug treatment for the long-term therapeutic benefit of dystonia patients. Likewise, discerning the mechanism of action of torsinA effector molecules will enhance compound optimization for therapeutic development and justification for clinical trials. Our initial evidence, suggesting that stabilization of torsinA protein levels following drug treatment correlates with functional rescue in both patient fibroblasts and knock-in mice, is indicative of one potential strategy.

Finally, although EOTD is a relatively rare disorder, given the endogenous expression and neuroprotective capacity of torsinA in DA neurons (Augood et al., 1998; Cao et al., 2005), it is intriguing to consider that small molecule enhancers of this protein might also be of potential therapeutic benefit for Parkinson's disease. Indeed, a neuroprotective role for torsins is further suggested by a report in flies, where targeted RNAi knockdown of *torp4a,* the only Drosophila torsinA homolog, causes progressive retinal degeneration (Muraro and Moffat, 2006). In this regard, the enhancement of native WT torsinA function may not only serve to overcome the neuronal deficit of mutant torsinA-associated EOTD, but might also be of benefit to the treatment of diseases resulting from defects in processing mutant proteins (e.g. cystic fibrosis), or perhaps of diseases involving hypersensitivity to ER-associated stress (e.g. Alzheimer's, Huntington's and Parkinson's diseases). Taken together, the results of this study further demonstrate the utility of cell systems and animal models for drug discovery research in facilitating the identification of small molecule therapeutics and accelerating their translational path to the clinic.

METHODS

C. elegans strains and protocols

Nematodes were maintained using standard procedures (Brenner, 1974). Transgenic lines were generated by microinjecting P_{unc-54}::Q82::GFP with P_{unc-54}::torsinA [strain UA38 (*baEx38*)], P_{unc-54}::torsinA(ΔE) [strain UA39 (*baEx39*)], or P_{unc-54}::torsinA and P_{unc-54}::torsinA(ΔE) [strain UA40 (*baEx40*)]. Each construct was injected at a concentration of 50 μg/ml, except for the P_{unc-54}::Q82::GFP + P_{unc-54}::torsinA + P_{unc-54}::torsinA(ΔE) line [strain UA40 (*baEx40*)], in which only 25 μg/ml of P_{unc-54}::torsinA and P_{unc-54}::torsinA(ΔE) were used. For each combination of constructs, at least three independent lines expressing extrachromosomal arrays were analyzed, and the most representative line was chosen for chromosomal integration using the method previously described (Caldwell et al., 2003). Following the integration, at least three independent integrated lines were analyzed and the most representative one was chosen for all the experiments throughout the paper. The resulting integrated lines include P_{unc-54}::Q82::GFP + P_{unc-54}::torsinA [strain UA41 (*baIn9*)], P_{unc-54}::Q82::GFP + P_{unc-54}::torsinA(ΔE) [strain UA42 (*baIn10*)], and P_{unc-54}::Q82::GFP + P_{unc-54}::torsinA + P_{unc-54}::torsinA(ΔE) [strain UA43 (*baIn11*)]. Two additional extrachromosomal array-expressing transgenic lines that were described previously {P_{dat-1}::GFP + P_{dat-1}::α-synuclein [strain UA18 (*baEx18*)] and P_{dat-1}::GFP + P_{dat-1}::CAT-2 [strain UA23 (*baEx23*)]} (Cao et al., 2005) were also integrated, and multiple independent lines were analyzed. The most representative one was chosen for all the related experiments throughout the paper and are designated P_{dat-1}::GFP + P_{dat-1}::α-synuclein [strain UA44 (*baIn11*)] and P_{dat-1}::GFP + P_{dat-1}::CAT-2 [strain UA45 (*baIn12*)]. Two additional strains used in this study were P_{glt-1}::*glt-1*(exons 1-3)::GFP and P_{hsp-4}::GFP(*zcIs4*)V (Calfon et al., 2002; Mano et al., 2007).

Chemical reagents

The Prestwick *C. elegans* chemical library was purchased from Prestwick Chemical Inc. (Illkirch, France). All candidate compounds used in assays beyond the primary screen and re-testing

were obtained from Sigma (St Louis, MO), except for metampicillin sodium salt, which was obtained from Microsource Discovery Systems Inc. (Gaylordsville, CT). Each compound was first dissolved in an appropriate solvent; compound solution was then added to pre-autoclaved media, with the volume of compound solution taken into account. All compounds were tested at an initial concentration of 0.5 mg/ml. The few compounds that were toxic to worms were tested at a concentration of 0.1 mg/ml or 0.025 mg/ml. Solvent controls were also analyzed. All worm plates were seeded with 100 µl of concentrated OP50 *E. coli*.

Worm screen with polyglutamine (Q82) torsinA activity readout

Five gravid adult worms corresponding to the appropriate transgenic line were placed onto each compound plate and grown at 20°C for approximately 3 days (supplementary material Fig. S1A). Thirty F1 worms at the L3 stage were counted for the number of Q82::GFP aggregates. All chemicals were analyzed twice, blinded, using a Nikon Eclipse E600 epifluorescence microscope using an Endow GFP filter cube. For the experiment described in supplementary material Fig. S5D,E, at least 30 transgenic worms expressing Q82 + torsinA WT/ΔE at the L2 stage were exposed to 0.5 mg/ml metampicillin, or water vehicle, until either the L4 or young adult stage, when they were analyzed for the number of Q82::GFP aggregates. This experiment was repeated twice.

Worm screen with -syn neuroprotection torsinA activity readout

Twenty adult worms were allowed to lay eggs for 5 hours on an appropriate control plate. After the progeny reached the late L4 stage (approximately 2 days after egg-laying), they were transferred to a fresh compound plate or control plate containing 0.4 mg/ml 5-fluoro-2′-deoxyuridine to inhibit progeny growth. Between 35-70 worms at the 7-day-old stage were scored for each independent experiment and three independent experiments were conducted (supplementary material Fig. S1B). The analyses of neurodegeneration were carried out according to the criteria described previously (Cao et al., 2005). These studies were conducted using a Nikon Eclipse E600 epifluorescence microscope using an Endow GFP filter cube.

Drug screening statistics

Statistical analyses for all *C. elegans* experiments were performed by *t* test using Systat. All average values of multiple independent experiments were expressed as mean ± S.E.M.

RNA isolation and semi-quantitative RT-PCR

Worms expressing $P_{glt\text{-}1}$::GFP were harvested and snap-frozen in liquid nitrogen. After total RNA isolation and cDNA preparation, semi-quantitative RT-PCR was performed, as described previously (Locke et al., 2006). Total RNAs from 50 L4-stage worms were isolated using Tri reagent (Molecular Research Center). Briefly, the worms were transferred into 10 µl of 1:10-diluted single worm lysis buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin and 60 µg proteinase K), mixed with 100 µl Tri reagent, and incubated at room temperature (RT) for 10 minutes. The samples were freeze-thawed five times using liquid N_2, vortexed with 10 µl of 1-bromo-3-chloropropane (Acros Organics) for 15 seconds, incubated at RT for 10 minutes, and centrifuged at 16,110 × ***g*** at 4°C for 15 minutes. The supernatant was transferred to a new RNase-free tube, mixed with 2 µl glycoblue (Ambion) and 50 µl of –20°C-chilled isopropanol, and incubated at –20°C overnight. After incubation, the supernatant was centrifuged at 16,110 × ***g*** at 4°C for 15 minutes, and discarded. The pellet was washed with 100 µl RNase-free 75% ethanol, and resuspended in 10 µl diethel pyrocarbonate (DEPC)-treated water. For RT-PCR using SuperScript III reverse transcriptase (Invitrogen) with oligo dT primers, the total RNAs were treated with amplification grade RNase-free DNase I (Invitrogen), as well as with RNase H (Invitrogen), following the manufacturer's protocol. PCR was then performed using Phusion polymerase (Finnzymes). The PCR products were separated by 0.8% agarose gel electrophoresis and visualized by GelRed staining (Biotium). The following primers were designed for the PCR: *glt-1* primer 1: 5′-CAC-TGT-CAG-TTT-GAC-CGC-C-3′, primer 2: 5′-TCG-TTC-ATG-GCG-ATT-GAG-TG-3′; *ama-1* primer 1: 5′-CGA-GTC-CAA-CGT-ACT-CTC-C-3′, primer 2: 5′-GAT-GTT-GGA-GAG-TAC-TGA-GC-3′.

***C. elegans* ER stress assay**

Worms expressing $P_{hsp\text{-}4}$::GFP (Calfon et al., 2002) were exposed to 0.5 mg/ml ampicillin or water solvent from hatching to the late L4 stage. ER stress was examined in late L4-stage animals that were transferred manually to nematode growth medium (NGM) plates spread with 9 µg/ml of tunicamycin in DMSO (MP Biomedicals) for 5 hours. Worms were then mounted on a 2% agarose pad and analyzed with a CCD camera (Photometrics CoolSnapHQ) on a Nikon (E800) microscope at 400× magnification. GFP intensities were measured in pixels and assigned arbitrary units (a.u.) in a 100 × 100 µm region immediately below the pharynx of each animal, in the anterior-most region of the intestine, using MetaMorph software (Molecular Devices). At least 50 animals were quantitated in three independent replicates.

Medicinal chemistry evaluation

BMU Consultants (Wenham, MA) performed this analysis. Briefly, initial compound alignments and visualization were generated using Discovery Viewer (Accelerys, San Diego, CA). Computational screening was accomplished with SciTegic Pipeline Pilot (Accelerys). All compounds in the *C. elegans* Prestwick library were included, as well as a library of penicillins, cephalosporins and quinolones, and the physicochemical properties were articulated by Lipinski's rules (Lipinski et al., 1997; Lipinski, 2000; Veber et al., 2002; Leeson and Davis, 2004). Compounds containing reactive or unstable moieties were excluded.

Fibroblast cell culture studies

Primary human skin fibroblasts from controls and *DYT1* patients were cultured as described (Hewett et al., 2007; Hewett et al., 2008). Self-inactivating lentivirus vectors encoded humanized Gluc (Prolume Ltd/Nanolight), downstream of the cytomegalovirus (CMV) promoter, followed by an internal ribosome entry site (IRES) and a cDNA for blue fluorescent protein, were packaged as described (Hewett et al., 2007; Sena-Esteves et al., 2004). To monitor Gluc secretion, cells were infected with these vectors to achieve >90% infection of cells then, at 72 hours post-infection, cells were re-plated in 48-well plates (5000 cells/well) (Hewett et al., 2007) in the presence

or absence of drugs at 5 μg/ml. Luciferase activity was monitored in conditioned, cell-free medium over the next 24 hours using a luminometer (Dynex) with 20 μM coelenterazine (Prolume Ltd/Nanolight) (Hewett et al., 2007). Experiments were repeated three times and the results represented as the mean ± S.E.M.

TorsinA quantification in human fibroblasts

To analyze the effect of ampicillin treatment on the levels of torsinA protein, fibroblasts from a control fibroblast line (HF71) and a *DYT1* patient (HF49) were plated in 6-well plates (60,000 cells/well) and treated with 6 μg/ml of ampicillin for 0, 24, 48 and 72 hours. Following ampicillin treatment, the cells were washed twice with PBS, pelleted by centrifugation, and the cell pellets were then resuspended in RIPA buffer (Hewett et al., 2008). Protein concentration was determined using a bovine serum albumin (BSA) standard (Bio-Rad, Hercules, CA). Twenty micrograms of the cell lysates were resolved by electrophoresis on a 10% SDS gel (Invitrogen). Proteins were transferred electrophoretically to nitrocellulose (Bio-Rad) and stained for protein with 0-2% Ponceau S (Sigma). Membranes were blocked in 10% non-fat milk powder in Tris-buffered saline containing Tween-20 (TBST; 150 mM NaCl, 50 mM Tris, pH 7.9, 1% Tween) for 1 hour. Blots were probed with antibodies to torsinA (D-M2A8; 1:100) (Hewett et al., 2003) and β-actin (1:3000; Sigma), diluted in TBST, and visualized with horseradish peroxidase (HRP) conjugated to a secondary sheep anti-mouse IgG antibody (1:8000; Amersham Pharmacia Biotech, Piscataway, NJ) using SuperSignal West Pico chemiluminescent substrate (Pierce). Four independent experiments were carried out for the control fibroblast line and three independent experiments for the DYT1 fibroblast line. For semi-quantitative analysis of protein levels, densitometry was performed on digital images obtained from western blots from all experiments using Quantity one-4.6.8 (Bio-Rad). Results were expressed with standard deviation, and significance was calculated by the Student's *t*-test (Excel).

Mouse studies

All of the mouse studies described below have been reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. A total of 20 *Dyt1* heterozygous ΔGAG knock-in male mice and 20 wild-type (control) littermates were tested at 146-164 days of age. Mice were housed under a 12-hour light and dark cycle. The control mice and the *Dyt1* ΔGAG knock-in mice were then both assigned randomly to two groups of 10 mice each, and coded. Ampicillin (10 mg/ml) solution was prepared fresh daily in saline using pyrogen-free water and injected intraperitoneally (100 mg/kg) on each test day (days 3 and 4) about 2 hours before the onset of the beam-walking test (Govindwar et al., 1984). The ampicillin dose was within the range used for patients (1 to 12 grams per person per day). Mice in one of the groups were injected with ampicillin, whereas mice in the other group were injected with vehicle solution (saline).

Mouse behavior studies

The beam-walking tests were performed, as described previously (Dang et al., 2005), by investigators who were blind to the genotypes and treatment. Briefly, the mice were trained to transverse a medium square beam (14 mm wide) in three consecutive trials each day for two days. They were then tested twice on the medium square beam and medium round beam (17 mm diameter) on the third day, and on the small round beam (10 mm diameter) and small square beam (7 mm diameter) on the fourth day. Hind paw slips on each side of the beam were recorded and analyzed. Statistics were performed using SAS/STAT Analyst software, as described previously (Yokoi et al., 2006). Significance was assigned at the $P<0.05$ level. The data of slip numbers in the beam-walking test for all four beams were analyzed together by logistic regression (GENMOD), with a negative binomial distribution using the generalized estimating equations (GEE) model (Allison et al., 2003). Control mice treated with vehicle solution were normalized to zero. To compare the effect of ampicillin treatment between the groups of mice, a dummy variable that combined genotype and ampicillin treatment was used, and contrasted to derive the *P* value between the vehicle and ampicillin group for each genotype.

TorsinA quantification in mouse brains

Protein extracts were prepared from 0.9% NaCl vehicle-treated *Dyt1* ΔGAG heterozygous KI mice ($n=4$) and their control littermates ($n=4$), and 100 mg/kg ampicillin-treated *Dyt1* ΔGAG heterozygous KI mice ($n=4$) and their control littermates ($n=3$). Two hours after the injections, the striata were dissected from the mouse brains and quickly frozen in liquid nitrogen. The striata were homogenized in 200 μl of ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4), 175 mM NaCl, 5 mM EDTA, CompleteMini (Roche)] and sonicated for 10 seconds. Lysis buffer containing 10% Triton X-100 (at a ratio of 9:1, homogenate:Triton solution) was added to the homogenates. The homogenates were incubated for 30 minutes on ice, and then the supernatants were obtained by centrifugation at 10,000 ***g*** for 15 minutes at 4°C. The protein concentration was measured by Bradford assay with BSA as standards. The homogenates were mixed with SDS-PAGE loading buffer and boiled for 5 minutes, incubated on ice for 1 minute, and then centrifuged for 5 minutes to obtain the supernatant. Forty μg of the samples were loaded on SDS-PAGE and the separated proteins were transferred to a Protran nitrocellulose transfer membrane (Whatman). The membrane was blocked in 5% milk in wash buffer [20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.1% (v/v) Tween 20]. The membrane was cut into two pieces to detect torsinA or β-tubulin, and each membrane was incubated overnight at 4°C with rabbit polyclonal torsinA antibody (ab34540; Abcam) or HRP-conjugated β-tubulin antibody (sc-5274 HRP; Santa Cruz) as a loading control. The membrane for torsinA was washed in the wash buffer and incubated with bovine anti-rabbit IgG-HRP (sc-2370; Santa Cruz) at room temperature for 1 hour, and then washed again. The membrane for β-tubulin was also washed with the wash buffer. The bands were detected by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). The signal was captured by Alpha Innotech FluorChem FC2. Western blot analysis was performed in triplicate. The density of the bands was quantified with Un-Scan-It gel (Silk Scientific) software. Molecular mass was estimated with Precision Plus Protein Standards All Blue (Bio-Rad). The density of the torsinA band was normalized to that of β-tubulin. The torsinA level in the vehicle-treated WT control mice was normalized to 100%, and the torsinA levels in other groups were compared by two-tailed Student's *t*-test. Significance was assigned at the $P<0.05$ level.

TRANSLATIONAL IMPACT

Clinical issue

Human movement disorders, including Parkinson's disease (PD) and dystonia, represent a substantial societal burden afflicting millions of people worldwide. Therapeutic options for these diseases are generally limited to treating symptoms rather than targeting the underlying molecular mechanisms. Early-onset torsion dystonia (EOTD) is characterized by involuntary muscle contractions and abnormal postures, which occur as a consequence of neuronal dysfunction. Mutation of the *DYT1* gene, encoding human torsinA, results in this dominantly inherited disorder. TorsinA is an ATPase that is thought to function like a chaperone in normal cells. EOTD is non-degenerative, as the affected neurons in the brains of patients remain intact. Furthermore, this disorder displays reduced penetrance, whereby only 30-40% of carriers exhibit symptoms. These combined features of the disease suggest that restoring torsinA function could be an excellent candidate for therapeutic intervention.

Results

Here, the authors used *C. elegans* in a screen to identify FDA-approved drugs that enhance torsinA activity. Using engineered nematode lines expressing human torsinA variants, they identified chemical enhancers of torsinA, including the common antibiotic ampicillin. In fibroblast cells isolated directly from *DYT1* patients, normal levels of torsinA function were restored by treatment with ampicillin. Finally, ampicillin treatment reversed a behavioral movement deficit within a well-characterized mouse model of *Dyt1* dystonia. This supports the development of ampicillin as a lead theraputic to benefit patients with torsinA defects, such as those with EOTD.

Implications and future directions

This work highlights the powerful utility of combining model systems to accelerate the translational path for human movement disorders, and reveals the potential for an unexploited new target for drug development. The capacity for ampicillin to activate torsinA function in a variety of bioassays, which include multiple cell types from several species, suggests a conserved molecular mechanism that may be reversed to restore neuronal function. Notably, the torsinA protein is highly expressed in human dopamine-producing neurons, where it is believed to serve a normally protective role. Therefore, the identification of small molecule enhancers of torsinA activity may be beneficial to many patients, in addition to those with dystonia, such as those with Parkinson's disease.

The identification of small molecule modifiers of torsinA function should enable the development of new drug therapy for neurological disease, such as dystonia. The long-term use of antibiotics is not desirable owing to its potential for creating antibiotic-resistant microorganisms and the eliminatination of necessary probiotics. Therefore, continued investigation should build from these findings to create a more suitable therapy than ampicillin that is a functionally related modifier of torsinA.

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Reagent sharing

Sharing of model organisms will be in compliance with NIH notice NOT-OD-04-042 (Sharing of Model Organisms for Biomedical Research). Short-term requests for nematode lines can be made directly to the Caldwell lab (kcaldwel@bama.ua.edu); long-term distribution will be facilitated by depositing them in the *Caenorhabditis* Genetics Center (CGC) (Caldwell lab strain code = UA). DYT1 fibroblast lines were established under IRB guidelines and are available to academic institutions by contacting Dr Breakefield (breakefield@hms.harvard.edu). Requesting investigators may be asked to defray costs of generating and distributing, or transferring, animal resources to appropriate repositories. Material transfers will be made following review and oversight of technology transfer offices.

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COMPETING INTERESTS

G.A.C. and K.A.C. serve as consultants to QRxPharma, Ltd, from whom they receive compensation and manage a sponsored research program for The University of Alabama.

AUTHOR CONTRIBUTIONS

The following represents the relative contributions of the authors to this publication: S.C. and Y.L. designed and performed research, analyzed data, and wrote/edited portions of the manuscript; J.W.H., F.Y., A.J.B. and F.C.N. designed and performed research, and analyzed data; J.L., A.C.B. and P.C. performed research and analyzed data; X.O.B., G.A.C. and K.A.C. designed research, analyzed data, and wrote/edited the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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