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A High-Content Screen Identifies Novel Compounds That Inhibit Stress-Induced TDP-43 Cellular Aggregation and Associated Cytotoxicity

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Abstract
TDP-43 is an RNA binding protein found to accumulate in the cytoplasm of brain and spinal cord from patients affected with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Nuclear TDP-43 protein regulates transcription through several mechanisms, and under stressed conditions, it forms cytoplasmic aggregates that co-localize with stress granule (SG) proteins in cell culture. These granules are also found in the brain and spinal cord of patients affected with ALS and FTLD. The mechanism through which TDP-43 might contribute to neurodegenerative diseases is poorly understood. To investigate the pathophysiology of TDP-43 aggregation and to isolate potential therapeutic targets, we screened a chemical library of 75,000 compounds using high-content analysis with PC12 cells that inducibly express human TDP-43 tagged with green fluorescent protein (GFP). The screen identified 16 compounds that dose-dependently decreased the TDP-43 inclusions without significant cellular toxicity or changes in total TDP-43 expression levels. To validate the effect, we tested compounds by Western blot analysis and in a Caenorhabditis elegans model that replicates some of the relevant disease phenotypes. The hits from this assay will be useful for elucidating regulation of TDP-43, stress granule response, and possible ALS therapeutics.

Keywords
amyotrophic lateral sclerosis, RNA granule, RNA binding protein, aggregation, high-throughput screen, protein synthesis

Introduction
Amyotrophic lateral sclerosis (ALS) occurs with an incidence of approximately 1/100,000.1 There is currently no therapy for ALS, and it is universally fatal. ALS presents with motor weakness in the distal limbs that rapidly progresses proximally.1,2 The causes of sporadic ALS are not known, but the identification of the major pathological aggregates accumulating in the spinal cord of patients with ALS represents a seminal advance for ALS research. Histological examination of ALS tissue reveals that TDP-43 is a major constituent protein within these aggregates in affected motor neurons in sporadic ALS.3 Mutations have been identified in TARDBP, the gene that encodes TDP-43, and they are associated with increased cytoplasmic inclusion formation in both familial and sporadic cases of ALS as well as in frontotemporal lobar degeneration.4,5 Notably, TDP-43 is the only protein in ALS that is both genetically and pathologically linked with sporadic ALS.

TDP-43 is an RNA/DNA binding protein that regulates transcription through several proposed mechanisms: RNA splicing, messenger RNA (mRNA) turnover, RNA trafficking, microRNA biogenesis, and DNA binding.6 Structurally, TDP-43 is divided into two functional domains: the
N-terminal domain containing RNA recognition motifs with nuclear localization and export signals and a C-terminal glycine-rich domain that mediates protein-protein interactions. The pathological mislocalization of TDP-43 results in an increase in cytoplasmic and nuclear aggregates and a concomitant loss of normal diffuse nuclear localization and function. Several studies have observed correlated cytotoxicity with increased protein expression of TDP-43, including associated neurotoxicity in primary rat cultures with ectopic expression of the disease-related mutant, A315T, of TDP-43. However, it is unclear whether the TDP-43–dependent pathological process results from a loss of normal function or a gain of toxic function. Although still debated, TDP-43 is thought to be highly regulated, including the ability to auto-regulate. Such regulation is suggested to be disrupted as a result from stress-induced mislocalization and accumulation of TDP-43 within stress granules (SGs). SGs are protein-mRNA aggregates that form in response to stressors and facilitate adaptive response of RNA translation toward cytoprotective proteins. The sequestration of TDP-43 into SGs likely yields an acute increase in TDP-43 as a result of depleted nuclear TDP-43. This feedback response further accelerates the accumulation of TDP-43 in SGs and depletes TDP-43 normal function.

The formation of cytoplasmic TDP-43 inclusions appears to be intimately linked to SG formation. Studies show that TDP-43 inclusions in human brain (as well as in cell culture) co localize with SGs and that agents inhibiting SG formation also inhibit formation of TDP-43 inclusions. These results suggest that neurodegeneration mediated by TDP-43 is linked to complex pleiotropic effects of protein translation dysregulation and stress granule biology. The putative relationship between TDP-43 and stress granule biology provides a novel approach for targeting TDP-43–related pathogenesis. Specifically, such an approach offers multiple opportunities to target TDP-43 pathophysiology by modifying a process that normally regulates SG formation, rather than direct physical disruption of protein aggregation by a small molecule. Identifying inhibitors of inclusion formation should elucidate whether the biochemical pathways regulating TDP-43 inclusion formation also regulate neurodegeneration. In addition, such inhibitors could offer a novel disease-modifying therapeutic approach in ALS.

In the present study, we performed a high-content screen for inhibitors of TDP-43 inclusion formation. We generated a Tet-Off inducible cell line that is stably transfected with a wild-type (WT) TDP-43::GFP fusion protein. TDP-43::GFP expression is induced by withdrawing doxycycline; the fusion protein exhibits a diffuse nuclear distribution under normal growth conditions. In contrast, TDP-43::GFP expression aggregates into cytoplasmic and nuclear inclusions under oxidative stress conditions. Using this robust distribution change as our readout, we screened approximately 75,000 chemicals from our general compound library. Sixteen compounds were found to reduce TDP-43::GFP inclusions without exhibiting toxicity in a dose-dependent manner. Hit compounds were tested for aggregation by Western blot analysis. A hit compound was then administered to transgenic Caenorhabditis elegans lines expressing WT TDP-43 and A315T TDP-43 as well as a nontransgenic line. The hit compound displayed neuroprotection and behavioral improvements in transgenic lines. Our data suggest that a phenotypic screen using high-content analysis is an effective method for discovering novel pharmacological inhibitors of TDP-43–related ALS pathophysiology. Furthermore, our hits can be used to dissect the molecular mechanism of the pathogenesis of TDP-43 inclusion formation, which should yield more targets against which alternative therapeutic agents for ALS can be developed.

### Materials and Methods

#### Reagents

C- terminally tagged WT human TDP-43::GFP was cloned into the pTRE2hyg vector (Clontech, Mountain View, CA). PC12 cells were obtained from Clontech as part of its Tet-Off cell line kit. Dulbecco’s modified Eagle’s medium (DMEM), horse serum, penicillin-streptomycin, hygromycin B, geneticin, trypsin-EDTA, 1× phosphate-buffered saline, and Hoechst 33342 were purchased from Life Technologies (Carlsbad, CA). Collagen I–coated 175-cm² vented flasks were from Becton Dickinson (Franklin Lakes, NJ). Collagen I–coated 384-well, optically clear, black-walled plates were purchased from PerkinElmer (Waltham, MA). Tet-Off plasmid, Tet system–approved fetal bovine serum (FBS), and doxycycline were purchased from Clontech. All other reagents, including sodium arsenite solution, were purchased from Sigma (St. Louis, MO).

#### Laboratory for Drug Discovery in Neurodegeneration Compound Library

The compound library consisted of approximately 75,000 small molecules, including compounds approved by the Food and Drug Administration (FDA), a purified natural products library, and compounds purchased from Peakdale (High Peak, UK), Maybridge Plc. (Cornwall, UK), Cerep (Paris, France), Bionet Research Ltd. (Cornwall, UK), Prestwick (Ilkich, France), Specs and Biospecs (CP Rijswijk, the Netherlands), ENAMINE (Kiev, Ukraine), Life Chemicals (Burlington, Canada), MicroSource Diversity System’s NINDS customs collection (Gaylordsville, CT), Chemical Diversity Labs (San Diego, CA), ChemBridge (San Diego, CA), and small molecules procured from various academic institutions. Compounds were selected from the different vendors by applying a series of filters,
including for clogP, polar surface area, and predicted solubility. All small molecules generally adhere to Lipinski’s rules (i.e., molecular weight <500, H-bond donors >5, H-bond acceptors >10, and logP <5) and contain a low proportion of known toxicophores (i.e., Michael acceptors and alkylating agents) and unwanted functionalities (i.e., imines, thiol, and quaternary amines) and have been optimized to maximize molecular diversity. Compounds for high-throughput screening (HTS) are stored as DMSO stocks at −20 °C.

**Generation of TDP-43::GFP Tet-Off Cell Line**

The rat PC12 cell line was used to generate the inducible TDP-43 cells. The cell line was generated by transfecting a Tet-Off inducible PC12 cell line (Clontech; this line stably expresses high levels of the tetracycline binding protein) with a WT human TDP-43::GFP construct with C-terminal GFP and selecting the stable transformants. The cell lines were developed from single-cell clones and maintained according to the manufacturer’s directions in DMEM containing 10% horse serum/5% calf serum, 1% penicillin-streptomycin, 100 μg/mL hygromycin B, and 100 μg/mL G418.

**Cell Culture**

Frozen stocks of PC12 cells with the TDP-43::GFP construct were plated in collagen coated T175 flasks and maintained in DMEM with 10% serum, 1% penicillin-streptomycin, hygromycin B, geneticin, and 1 μg/mL doxycycline for 24 h at 37 °C, 95% relative humidity, and 10% CO2. Cells were trypsinized, passaged and incubated in induction media (no Dox) at 37 °C and 10% CO2 for 2 days and passaged again, for a total induction time of 3 days. After the second passage, Tet-Off TDP-43::GFP induced cells were passaged and plated into 384-well assay plates at 3000 cells/well with 50 μL of induction media using a Multidrop automated liquid handler (Thermo Fisher Scientific, Waltham, MA). Gas-permeable seals were used to reduce any evaporation effects. Cells were then treated with compound and incubated for 18 h before being fixed, labeled, and imaged (details below).

**TDP-43::GFP Aggregation Assay and Compound Addition**

After the assay plates were incubated overnight, compounds from the Laboratory for Drug Discovery in Neurodegeneration (LDDN) library were transferred using a robotic liquid handler (Beckman Coulter, Brea, CA) into columns 1 to 22 of each plate with a final concentration of 1 μM. DMSO was added to columns 23 and 24 with a final concentration of 0.1%. Plates with compounds were incubated for 1 h at 37 °C. Sodium arsenite was then added to columns 1 to 23 with a final concentration of 15 μM and incubated for 18 h at 37 °C, 95% relative humidity, and 10% CO2. Columns 23 and 24 of each assay plate represent negative and positive TDP-43::GFP aggregation inhibition controls, respectively.

**Image Acquisition and Analysis**

Cells were fixed with 4% paraformaldehyde for 20 min and washed three times with phosphate-buffered saline (PBS). The cells were labeled with 1 μg/mL Hoechst stain to visualize the nuclei. Plates were imaged on the IN Cell Analyzer 1000 (GE Healthcare, Piscataway, NJ). In each well, images were acquired in five preselected fields over two channels, with λ = 360-nm excitation/λ = 535-nm emission for Hoechst and λ = 474-nm excitation/λ = 535-nm emission for TDP-43::GFP fusion protein, at 200-ms and 20-ms exposure times, respectively. Image stacks were batched and analyzed using IN Cell Workstation software (GE Healthcare). For the feature extraction protocol, cells were segmented using the multitarget analysis algorithm, beginning by segmenting nuclei with a minimum area of 22 μm² with a sensitivity setting of 64. A collar of 5 μm around the nucleus defined the whole cell. The TDP-43::GFP labeling was separated into two distinct phenotypes: diffuse nuclear and cellular puncta (aggregates). For diffuse nuclear TDP-43::GFP segmentation, granule diameter settings of 3 μm minimum and 11 μm maximum with scaled bias toward larger objects to prevent inappropriate segmentation and a sensitivity setting of 91 were used. For cellular puncta TDP-43::GFP segmentation, granule diameter settings of 1 μm minimum and 5 μm maximum with scaled bias toward small objects and a sensitivity setting of 79 were used. Feature outputs include cell count; diffuse nuclear TDP-43::GFP count, mean area, and intensity; inclusion fraction (puncta TDP-43::GFP count, mean area, and intensity; number and percent of TDP-43::GFP positive cells; and number and percent of cells with TDP-43::GFP aggregates. TDP-43::GFP positive cells were identified as cells having the presence of diffuse nuclear TDP-43::GFP. Aggregate positive cells were identified as cells having the number of TDP-43::GFP puncta in a cell greater or equal to 1.

**Hit Selection, Confirmation, and Validation**

A compound was considered a “hit” and selected for validation if it increased inhibition >3 times the standard deviation of the DMSO control wells. The threshold for hits was defined as follows: (Inclusion fraction for sodium arsenite control) – 3*SD = 0.36. The inclusion fraction was converted to percent inhibition by dividing the inclusion fraction of samples by the mean Ars control and multiplying by 100 (~45). The 114 hits that were greater than three
standard deviations from control were then retested and subjected to 5-point dose-response curves in triplicate from the original library stock. Twenty-two of the 114 confirmed hits were initially selected because they reduced TDP-43 inclusions by more than three standard deviations, showed less than 20% toxicity based on cell counts, and represented a distinct chemical class. Finally, selected confirmed hits were reordered from the commercial suppliers to validate the hits, and a 12-point dose-response series of 3-fold dilutions ranging from 0.01 to 30 μM final concentration was tested in quadruplicate in the HTS assay to generate EC_{50} values for each compound.

Western Blot Analysis

Cells were grown on collagen-coated 10-cm plates, scraped, and homogenized in lysis buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2% sodium dodecyl sulfate [SDS], PMSF, and Halt protease inhibitor cocktail [Thermo Fisher Scientific] and phosphatase inhibitor cocktail [PhosSTOP; Roche, Indianapolis, IN]). For cell fractionation, lysates were combined 1:1 with RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5 mM sodium deoxycholate) with 1× Halt protease inhibitor cocktail (Thermo Fisher Scientific), 1× phosphatase inhibitor cocktail (PhosSTOP; Roche), and 2% Triton X-100 and sonicated. Protein concentration was assessed by BCA assay (Pierce, Rockford, IL). Following Western blotting, membranes were incubated with anti-rabbit human-specific monoclonal TDP-43 antibody (10782-2-AP, Proteintech, Chicago, IL).

C. elegans Culture and Experimentation

C. elegans were cultured on seeded nematode growth media (NGM) plates at 15 °C or 20 °C, and all experiments were performed at 20 °C. The C. elegans lines expressing human WT or A315T hTDP-43 were utilized. The labels are as follows: CK405 (WT hTDP-43 high expresser line) and CK426 (A315T hTDP-43 low expresser line), as defined previously by immunoblot analysis. All nematode experiments were performed using age-synchronized animals, by either bleaching (for experiments requiring large numbers of nematodes) or timed egg laying (for experiments requiring small numbers of nematodes). Compounds were added to solidified NGM plates by spreading compound in an aqueous solution containing 10% DMSO, such that the final concentration of DMSO was 0.1%. The solution was allowed to dry for 1 h before addition of the OP50 bacteria as the food source.

For speed assessment, photos of plates containing at least 15 C. elegans were automatically taken every 6 s for different time spans (N2: 1 min, CK405: 2 min, and CK426: 5 min). Using the AxioVision software (Carl Zeiss Microimaging, Thornwood, NY), a movie could be obtained from the single pictures, and the covered distance of each nematode could be measured. By dividing the distance (mm) and the measured time (min), the speed (mm/min) was obtained. For each condition, two replicate plates each containing 10 to 30 nematodes were used. The average speed was finally calculated from data of all C. elegans, where values that exceeded the mean value ± 3-fold standard deviation were excluded.

For quantifying motor neurons, in CZ-1200 (non-TDP-43), the nerve cord is continuously connected and the normal number of 19 motor neurons can be seen. CK476 expressing mutant TDP-43 show dramatic degeneration of motor neurons. The damage includes loss of neuron cell bodies and the extensions of gaps in neurites of the nerve cord. Age-synchronized nematodes were cultured with compound or vehicle, and surviving neuron cell bodies and neurites were scored. Studies were performed using vehicle-treated CK476 (n = 24) and compound-treated CK476 (n = 20).

Statistics

Statistical significance was calculated using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA) and the Student t test to determine when a p value of 0.05 or better had been achieved between groups. The values are indicated in the text and figures. For HTS, Z’ calculations rely on both a maximum and minimum signal, in this case with and without sodium arsenite treatment. In addition, reproducibility across a group of plates and assay days as measured by the mean and standard deviation of the signal is included in the results.

Results

Tet-Off–Engineered PC12 Line Expresses Nuclear TDP-43

The goal of this screen was to identify compounds that can inhibit the formation of TDP-43 aggregates in cells. To accommodate cell culture conditions (e.g., multiple passages, freeze-thaw cycles, and automation), PC-12 cells were selected for engineering. It was important that the expression system be inducible, because high levels of WT human TDP-43 have been shown to induce cell toxicity. We generated a TDP-43::GFP fusion protein that we cloned into the Tet-Off system. We chose to use a Tet-Off system to eliminate any interference that doxycycline (Dox) would have on the library compounds if a Tet-On system was used. Figure 1A represents a schematic diagram of the cloning vector that was used in this screen. The cells stably expressed the construct and were developed from single-cell clones. As the cells expanded in the presence of Dox, the cells did not express...
detectable levels of TDP-43::GFP (Fig. 1B). In contrast, when Dox was removed, the cells robustly expressed TDP-43::GFP with diffuse nuclear distribution (Fig. 1C). Despite being clonal, TDP-43::GFP expression varied among cells; the varied expression occurred in multiple clonal lines and was still present after recloning of the line from individual cells. Although the reason for this variation is not fully known, it could be caused by cell state–specific autoregulation or by stochastic control of TDP-43 expression. Some Tet regulators that were found from our screen (Suppl. Fig. S2) induced TDP-43 in all cells demonstrating the presence of the transgene (data not shown). In the presence of normal growth conditions, TDP-43::GFP formed very few aggregates (Figs. 1B and 2A,B). This condition represents a normal, WT expression distribution of TDP-43.

**Sodium Arsenite Induces a Robust TDP-43::GFP Aggregation Response**

TDP-43 is an RNA binding protein with a glycine-rich domain. Glycine-rich domains are hydrophobic and have been shown to mediate reversible aggregation into insoluble macromolecules. Stress granule (SG) formation is one such aggregation event. In response to cellular stressors, SGs mediate transcriptional alterations by sequestering nonessential transcripts. TDP-43 has been associated with SGs in cell culture models of ALS. We hypothesized that TDP-43::GFP would aggregate in response to sodium arsenite, which is known to strongly induce SGs through a pleiotropic mechanism that involves oxidative and other stress pathways. To test this, we exposed the engineered cell line for 18 h to sodium arsenite, which produces the arsenic oxoanion. We also tried other inducers, such as hydrogen peroxide and nutrient deprivation, as previously described. However, only sodium arsenite delivered a response that was sufficiently consistent and robust (occurring in at least 70% of cells) to be feasible for a high-throughput assay. In the presence of 15 µM sodium arsenite, TDP-43::GFP formed nuclear and cytoplasmic aggregates (Fig. 1D,E). The inclusions were evident in both the nucleus and the cytoplasm, although in this cell model, we saw more inclusions in the nucleus than in the cytoplasm. Immunocytochemical experiments demonstrated that these SGs co-localized with the SG marker TIA-1, and acute treatment (1 h) with classic SG dispersal agents, such as cycloheximide (50 µg/mL) or emetine (5 µM), reversed SG formation (data not shown). Immunocytochemistry with anti–TDP-43 antibody produced results that were identical to those observed with the TDP-43::GFP construct, which is a similar result that was observed previously. Although nuclear localization is atypical for stress granules, nuclear TDP-43 inclusions are readily detectable in frontotemporal dementia. This phenotype recapitulates the distribution of TDP-43 associated with disease pathology. We examined the dose response to sodium arsenite of multiple parameters, including the nuclear fraction of TDP-43::GFP, the inclusion-associated TDP-43::GFP, and the total cell count. Aggregation count refers to the mean number of aggregates per cell in a well. With increasing sodium arsenite concentration for 18 h, the inclusion fraction of TDP-43::GFP increased relative to the nuclear fraction (Fig. 2A). In total, 15 µM sodium arsenite was found to induce a significant increase in TDP-43::GFP aggregation fraction with minimum toxicity (Fig. 2A,B). Concentrations above 15 µM sodium arsenite elicited an aggregation response with significant cytotoxicity (Fig. 2A). The presence and absence of...
Sodium Arsenite Induces TDP-43::GFP Aggregation Scales for Screening

After the Tet-Off TDP-43::GFP expression control conditions were established, we proceeded to evaluate the suitability of the procedure for HTS. We prepared test plates in the 384-well format to measure signal strength, interwell consistency of signal, and reproducibility in sodium arsenite– and vehicle (DMSO)–treated cells. Cell density in the range of 3000 to 4000 per well was found to be optimal (data not shown). The TDP-43::GFP aggregation signal increased approximately 5-fold with sodium arsenite treatment (Fig. 2B). The well-to-well variation was approximately 20%. We measured Z’ factor to assess whether changes in aggregation interwell variation can accurately distinguish “hits” in a large number of test compounds (Fig. 2C). For Z’ factor calculations, we used cells treated with and without 15 μM sodium arsenite. The average Z’ factor calculated for our test plates was 0.23, and this was consistent for HTS. A Z’ factor >0.5 is typically considered suitable for HTS. However, in phenotypic assays such as this, we have found that a Z’ factor above 0.2 is acceptable to identify hits. To test the reproducibility of the assay, we screened a set of 2000 compounds in triplicate. The TDP-43::GFP inclusion fractions of the triplicates were plotted against each other. Although the inclusion fraction is quite variable at high levels (greater than 0.7) of inclusions, conditions that reduce the inclusion fraction below 0.6 display robust reproducibility (Suppl. Fig. S1).

High-Content Screen Identifies Compounds Inhibiting Sodium Arsenite–Induced TDP-43::GFP Aggregation

PC12 cells expressing TDP-43::GFP were plated as described in the Materials and Methods, and compounds were added to columns 1 to 22 of each 384-well plate at a final concentration of 1 μM and 0.1% DMSO. In total, 75,000 compounds were screened in singletons. Columns 23 and 24 were spotted with 0.1% final concentration of DMSO. After an hour, 15 μM sodium arsenite was added to all columns except column 24; plates were then incubated for another 18 h. Cells were fixed and imaged on the IN Cell Analyzer (GE Healthcare). IN Cell Workstation software was used to analyze multiple features of the image sets. Multivariate readouts were separated into two fractions: the TDP-43 fraction, which represents the diffuse nuclear distribution of TDP-43::GFP, and the inclusion fraction, which represents the puncta nuclear and cytoplasmic distribution of TDP-43::GFP. Within each fraction,
mean count/cell (“Count”), mean area of the feature (“Mean Area”), and intensity of the TDP-43::GFP signal (“Intensity”) were quantified. Total cell count was also included in the readout. The inclusion fraction was used to identify putative hits. The inclusion fraction was converted to percent inhibition as described in the Materials and Methods. Once compounds were identified to reduce the inclusion fraction, they were filtered against other measurements to eliminate false positives. Hits were identified as compounds reducing the inclusion fraction without affecting the TDP-43 fraction and cell count. Compounds were considered toxic if they induced greater than 20% cell loss. Examples of the comprehensive readouts are represented in Table 1. In this table, where five hits are examined, highlighted values represent statistically significant (>3 SD) reductions compared with sodium arsenite control. Note in this selection of compound conditions, all compounds reduced the inclusion fraction. In contrast, compound LDN-0000020 reduced all the TDP-43::GFP measurements below the sodium arsenite control, suggesting that this compound inhibited the transcription or translation of TDP-43::GFP rather than targeting the aggregation. A set of six compounds with this profile were identified as doxycycline analogues (Suppl. Fig. S2). Compound LDN-0002741 also may have a similar mechanism of action, as it also reduced the TDP-43::GFP fraction as well as the inclusion fraction. Thus, of the selected compounds in Table 1, compounds LDN-0002590, LDN-0000827, and LDN-0001080 exhibited a desirable hit profile. In total, 114 hits that met criteria were then confirmed in a 5-point dose response from original compound stocks. Twenty-two of the confirmed hits were selected based on potency and structure to represent 10 different diverse chemical classes and then were validated in a 12-point dose-response curves (Fig. 3). Supplementary Table S1 lists the hit compounds, IC₅₀s, and maximum TDP-43::GFP aggregation inhibition.

Western Blot Confirms That Hit Compound Inhibits TDP-43::GFP Aggregation

To test whether the lead compounds modulate aggregation of TDP-43, we examined the effects of compound LDN-0130436 on spontaneous and sodium arsenite–induced TDP-43::GFP aggregation by immunoblot using the TDP-43 inducible PC12 cell line (Fig. 4). For these studies, TDP-43 was induced and the cells were treated with sodium arsenite (0.5 mM, 1 h) ± LDN-0130436 (3.5 μM). The cells were then lysed, fractionated into soluble/insoluble protein, run on a gel, and then immunoblotted using anti–TDP-43 antibodies. We observed distinct bands representing TDP-43::GFP at a predicted molecular weight (~75 kDa) and a higher molecular weight (~150+ kDa), which increased in the presence of sodium arsenite. Cells treated with LDN-0130436 exhibited a striking reduction in levels of aggregated TDP-43 either with sodium arsenite (Fig. 4, lane 6) or under basal conditions (Fig. 4, lane 3). Treatment with LDN-0130436 also reduced a lower molecular weight TDP-43 band, which might be a TDP-43 cleavage fragment similar to that observed in patients with ALS (Fig. 4). We also fractionated the cell lysates and demonstrated that LDN-0130436 causes an equally impressive translocation of TDP-43::GFP from the insoluble to the soluble fraction. We observed that this higher band is significantly reduced with LDN-0130436 with little effect on the lower band, suggesting that, indeed, the hit compound is reducing the aggregation of TDP-43::GFP.

Table 1. Multiaspect Readouts for Hit Profiling.

<table>
<thead>
<tr>
<th>Plates 1–10: Well</th>
<th>Total TDP-43 Signal: Cell Count Fraction</th>
<th>Mean Area</th>
<th>Intensity</th>
<th>TDP-43 Inclusion Signal: Mean Inclusion Count per Cell</th>
<th>Mean Area</th>
<th>Intensity</th>
<th>Total Cells in Four Fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDN-0000020</td>
<td>0.252</td>
<td>84.247</td>
<td>157.8</td>
<td>0.048</td>
<td>39.886</td>
<td>309.94</td>
<td>516</td>
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<tr>
<td>LDN-0002741</td>
<td>0.226</td>
<td>102.84</td>
<td>398.13</td>
<td>0.186</td>
<td>49.998</td>
<td>552.35</td>
<td>641</td>
</tr>
<tr>
<td>LDN-0002590</td>
<td>0.603</td>
<td>97.211</td>
<td>354.28</td>
<td>0.173</td>
<td>42.244</td>
<td>680.08</td>
<td>416</td>
</tr>
<tr>
<td>LDN-0000827</td>
<td>0.296</td>
<td>93.525</td>
<td>339.82</td>
<td>0.236</td>
<td>46.666</td>
<td>521.78</td>
<td>609</td>
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<tr>
<td>LDN-0001080</td>
<td>0.39</td>
<td>86.995</td>
<td>314.6</td>
<td>0.231</td>
<td>48.292</td>
<td>532.22</td>
<td>520</td>
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<tr>
<td>Ars DMSO control</td>
<td>0.4463</td>
<td>93.523</td>
<td>417.7</td>
<td>0.4154</td>
<td>50.456</td>
<td>584.5</td>
<td>370.75</td>
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</table>
Figure 3. Hit validation 12-point dose response. Sixteen hits with associated dose-dependent inhibition of TDP-43::GFP inclusion fraction (▲). Cell count (■) and nuclear-associated TDP-43::GFP (▼) are also represented. Data represent mean ± standard deviation; n = 4.
Figure 4. LDN-0130436 inhibits TDP-43::GFP aggregation. Immunoblot with anti-GFP, showing expression and aggregation of TDP-43::GFP. The spontaneous aggregation of TDP-43 (lane 2) is increased in the presence of 15 μM sodium arsenite (lanes 5–8). Lead compound LDN-0130436 inhibits aggregation in both conditions with little effect on TDP-43::GFP expression (lanes 3 and 6).

Hit Compound Exhibits Neuroprotective Actions in a TDP-43 Transgenic C. elegans Nemotode Model

Next we tested each of the 16 lead compounds for efficacy in a transgenic model of TDP-43 toxicity using C. elegans. We used C. elegans lines expressing TDP-43 (WT and A315T). A315T is a missense mutation in the gene that segregates with all affected members of an autosomal dominant family with ALS and not found in 1505 healthy control subjects.5 These C. elegans lines have been reported to exhibit motor neuron loss and associated behavioral deficits.17 C. elegans are frequently less sensitive to small molecules because of the thick cuticle, which resists penetration of compounds. Hence, we used doses that were 10 to 200 times the IC_{50} observed with cell lines, because C. elegans tend to be much less sensitive to exogenous compounds than cells grown in culture. We tested the efficacy of the optimized lead compounds in ameliorating cell loss and motor dysfunction associated with TDP-43 expression by treating at birth and measuring responses at adult day 1. The nematode lines were hatched on agar plates containing varying doses of the lead compounds. One of the compounds, LDN-0130436, was found to increase movement in the CK406 line expressing WT TDP-43 while not affecting the nontransgenic N2 line (Fig. 5). One other compound, LDN-0125735, also increased movement but did so to similar degrees in both the N2 and CK406 lines (data not shown). As expected, transgenic lines also displayed a reduction of motor neurons (Fig. 5A,B). In the presence of LDN-0130436 (34.8 μM), we observed protection against TDP-43–mediated cell loss (Fig. 5c). Furthermore, the behavioral deficits displayed by transgenic worms appeared partially or wholly ameliorated by LDN-0130436 (Fig. 5D). Notably, the compound had no measurable effect on nontransgenic worm cells (data not shown) or behavior (Fig. 5D). Thus, the observed effects of LDN-0130436 in our C. elegans assay are both strong and protective against TDP-43–mediated toxicity.

Discussion

ALS is a chronic neurodegenerative disease associated with motor neuron loss. This cell loss is hypothesized to result, in part, from a mislocalization of the TARDBP gene product TDP-43. TDP-43 has been implicated in both familial and sporadic ALS. Its expression is highly regulated, and its mislocalization confers pathogenic phenotypes in models of ALS. We and others have shown that modifications of TDP-43 expression in cell culture and in whole organisms yield phenotypes similar to ALS tissue and can cause cytotoxicity.7,8 One means by which TDP-43 is mislocalized is its sequestration into stress granules. To investigate the pathophysiology of TDP-43 aggregation into stress granules, we developed a high-content screen to identify compounds that inhibit the formation of TDP-43 aggregates.

We have developed an inducible cell line that expresses WT TDP-43 and robustly develops inclusions in response to the stressor, sodium arsenite. This approach obviates potential toxicity that is commonly observed with stable overexpression of TDP-43. The inducible expression of TDP-43::GFP and sodium arsenite–mediated aggregation was miniaturized to accommodate a 384-well plate format. After much effort to optimize, we could not improve the variability reflected in the Z′ factor (0.23) and attribute it to the biology of TDP-43, which is highly regulated by the cell, as evidenced that too much or too little TDP-43 is detrimental. With our validation assay using sets of plates in triplicate, we could demonstrate good reproducibility and went forward with the screen. In the end, we found robust, reproducible hits that have been validated in additional assays. We demonstrated the validation of one lead compound, LDN-0130436, by examining its effects on TDP-43 aggregation by Western blot analysis. Furthermore, we showed that this compound could be neuroprotective in transgenic C. elegans expressing WT and A315T TDP-43 with no effect on nontransgenic worms. Our data support the hypothesis that TDP-43 inclusions form in conjunction with the SG pathway and that inhibitors of SG formation will also inhibit TDP-43 inclusion formation.8 We are currently exploring this relationship in more detail.

Key to the success of this screen was the utilization of high-content analysis. Because the primary readout was aggregation, not total expression, an imaging assay was
necessary for screening. In addition, high-content analysis enables multivariate analysis that is necessary for physiologically relevant screens for neurological diseases.\textsuperscript{18} In this study, we assessed the aggregation expression profile of TDP-43, the diffuse nuclear expression of TDP-43, and the total cell number. The multivariate readout gave us the ability to define the fingerprint for a desired phenotype and filter potential false-positive hits early in the screening process.

The lead compounds provide a basis to explore the biology of TDP-43 and stress granules as a potential therapy for ALS. Further work will need to be completed before identifying whether the mechanism of action of the lead compounds targets stress granule formation, as well as whether the compounds are specific for TDP-43, or work via a shared mechanism with other proteins that are known to form aggregates, and have neuroprotective potential in mammalian models.

It is feasible that a cluster of compounds will exhibit inhibitory effects on TDP-43 without inhibiting the stress granule response, while other compounds will have a more generalizable inhibitory effect on aggregation or other basic cell biological processes implicated in TDP-43–associated toxicity. Such compounds will be useful tools to dissect the functional role of SGs in TDP-43–associated pathogenesis and possibly help to identify novel targets in this pathway.

Recent reports provide potential pathways that may be targeted by our lead compounds. Cell biological processes have been described to mediate TDP-43 toxicity: stress signaling response, protein degradation, and transcriptional regulation. TDP-43 phosphorylation has been implicated in aggregation and can result in response to oxidative stress.\textsuperscript{19} In a kinome-based RNAi screen, CDC7 was found to directly phosphorylate TDP-43 under oxidative stress conditions.\textsuperscript{20} The authors demonstrate that pharmacological inhibition prevented TDP-43 phosphorylation and associated cytotoxicity.\textsuperscript{20} Other stress-related kinases have also been associated with TDP-43 toxicity.\textsuperscript{21} By targeting the stress kinase, JNK, and its downstream effector, c-Jun, researchers report that they could modulate the TDP-43–associated toxicity.\textsuperscript{22} Interestingly, TDP-43 toxicity and SG formation were inhibited by targeting ERK, which the authors suggest is a response to oxidative stress.

Figure 5. LDN-0130436 protects against TDP-43–associated cell loss. (A) Transgenic Caenorhabditis elegans expressing unc-25::GFP expressing GFP in the GABA-ergic motor neurons. (B) Transgenic C. elegans that also express TDP-43 (A315T) exhibit reduced unc-25::GFP expression GFP in the GABA-ergic motor neurons by adult day 2, treated with vehicle (0.58% DMSO). (C) unc-25::GFP/ TDP-43 (A315T) C. elegans lines treated with LDN-0130436 from birth (34.8 µM in 0.58% DMSO). Note the rescue of neurons in the worm exposed to compound. (D) Behavioral data from nontransgenic (N2), wild-type (WT) TDP-43 transgenic, and A315T TDP-43 transgenic worms exposed to increasing doses of LDN-0130436. High doses of compound improve the behavioral deficits in TDP-43 transgenic worms. No significant change in nontransgenic worm behavior is observed. Data represent mean ± standard deviation; \( n = 4. ** p < 0.05. \)
stress-induced downregulation of mitochondria activity.23 Dysfunction of two cellular catabolic processes, ubiquitination and autophagy, has been explored as possible mediators of TDP-43 aggregation. Ubiquitin-2, a component of the ubiquitin pathway, was recently shown to bind TDP-43 directly.24 Disrupting this binding elicited a TDP-43 aggregation response. Similarly, modifications of autophagy have demonstrated efficacy in modulating TDP-43 toxicity. Four different compounds—rapamycin, spermadine, carbamazepine, and tamoxifen—each of which activates autophagy, all demonstrated neuroprotection in TDP-43 transgenic mice; in addition, rapamycin elicited inhibition of TDP-43 aggregation.25 Consistent results were reported when modifications of TDP-43 autophagic clearance via the Cdc/Hsp90 complex were found to be protective.26 When induced pluripotent stem cells derived from familial ALS patients with TDP-43 mutations were screened for compounds inhibiting motor neuron–associated pathology, the antibiotic anacardic acid was found to be protective.27 The targets neuroprotective by inhibiting TDP-43 transcription, leading to ated pathology, the antibiotic anacardic acid was found to be screened for compounds inhibiting motor neuron–associated to familial ALS patients with TDP-43 mutations mon theme among neurodegenerative diseases. Phenotypic larly those related to stress signaling and autophagy. inhibitors as potential hits. Likely candidates for drug development in models of neurodegeneration. Cytotoxicity related to aberrant aggregation is a common theme among neurodegenerative diseases. Phenotypic screens to identify conditions that inhibit aggregation could be useful tools for drug discovery in neurodegeneration.28 The approach used in this screen serves as a proof of concept that a high-content imaging screen can successfully identify compounds that inhibit the process of protein aggregation and that such compounds can confer neuroprotection in models of neurodegeneration.

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