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An Allosteric Modulator of RNA Binding Targeting the N-Terminal Domain of TDP-43 Yields Neuroprotective Properties

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.0c00494>.

Supplementary Table 1 (XLSX)

Description of the methods, spectral data, and all supplementary figures (PDF)

The authors declare no competing financial interest.

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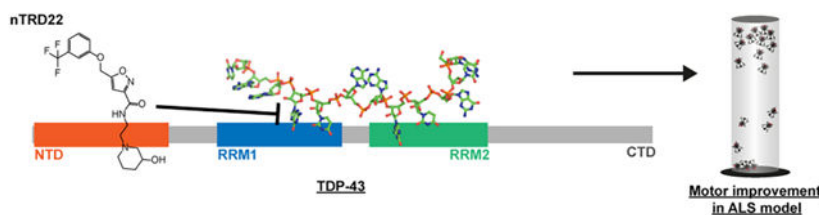
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Abstract

In this study, we targeted the N-terminal domain (NTD) of transactive response (TAR) DNA binding protein (TDP-43), which is implicated in several neurodegenerative diseases. *In silico* docking of 50K compounds to the NTD domain of TDP-43 identified a small molecule (nTRD22) that is bound to the N-terminal domain. Interestingly, nTRD22 caused allosteric modulation of the RNA binding domain (RRM) of TDP-43, resulting in decreased binding to RNA *in vitro*. Moreover, incubation of primary motor neurons with nTRD22 induced a reduction of TDP-43 protein levels, similar to TDP-43 RNA binding-deficient mutants and supporting a disruption of TDP-43 binding to RNA. Finally, nTRD22 mitigated motor impairment in a *Drosophila* model of amyotrophic lateral sclerosis. Our findings provide an exciting way of allosteric modulation of the RNA-binding region of TDP-43 through the N-terminal domain.

Graphical Abstract



Transactive response (TAR) DNA binding Protein-43 (TDP-43), which is a critical factor in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD), is involved in almost all aspects of RNA metabolism.^{1,2} TDP-43 consists of an N-terminal domain (NTD), two RNA recognition motifs (RRM1 and RRM2), and an unstructured glycine-rich domain.

The NTD is responsible for TDP-43 subcellular localization, since it contains a nuclear localization signal (NLS)³⁻⁶ and one mitochondrial targeting sequence (M1).⁷ TDP-43 NTD has been shown to form dimers that can assemble into reversible higher-order oligomers, required for splicing activity⁸⁻¹¹ and contributing to liquid–liquid phase separation.^{8,11} Several studies have hinted at the ability of TDP-43 NTD to serve as a scaffold for nucleic acid binding and to contribute to specificity toward certain nucleic acid sequences,¹²⁻¹⁴ although a nucleotide binding interface of the NTD remains to be determined.

In a previous study,¹⁵ we targeted the RNA-binding domain (RRM1) of TDP-43 and found a compound, rTRD01, that was bound to TDP-43, partially disrupted RNA binding, and improved locomotor defects in a *Drosophila* model of ALS. The current study is distinct because it targets the N-terminal domain and identifies an indirect effect on the RNA-binding domain. Based on the potential role of TDP-43 NTD in nucleic acid binding, as well as protein aggregation, we sought to target the NTD with small molecules using *in silico* docking. Neither X-ray structures (PDB 5mdi⁸) nor NMR structures (PDB 2n4p¹²) of TDP-43 reveal any obvious surface pocket(s) or large cleft(s) capable of accommodating small molecules. The program SiteMap^{15,16} was used to determine if residues directly implicated in dimerization or residues nearby could form a druggable pocket for binding of small molecules. A druggable pocket was identified by Sitemap on the NMR structure, pointing to the involvement of residues Ser48, Ala66, and Asn70 (Supplementary Figure 1A in the Supporting Information). A slightly different site was found on the X-ray structure and surrounded the following residues: Tyr43, Leu56, and Asp65 (see Supplementary Figure 1B in the Supporting Information).

A 50 000-compound library was then docked onto a grid of 6 Å surrounding the Sitemap pockets, using Glide's virtual screening workflow. Even though the initial pockets were different on the NMR and crystal structure, compounds were docked around Ser48, Ala66, and Asn70 in both structures (Figure 1). A total of 20 compounds were chosen based on score and other energy-related terms: nTRD09–nTRD18 for the X-ray structure and nTRD19–nTRD28 for the NMR structure (Supplementary Table 1 in the Supporting Information). Visual inspection of the docking poses was implemented to remove unrealistic poses as they might result in high scoring.¹⁶

Saturation transfer difference nuclear magnetic resonance (STD-NMR) spectroscopy was then used to screen the binding of the compounds to TDP-43₁₋₂₆₀. As a negative control, binding to TDP43₁₀₂₋₂₆₉, a construct lacking the NTD, was also determined (Supplementary Figure 2A in the Supporting Information). We observed nTRD12–nTRD14 and nTRD22, nTRD25–nTRD28 as positive hits on TDP-43₁₋₂₆₀ that did not bind to TDP43₁₀₂₋₂₆₉ (see Supplementary Figures 2B-2D in the Supporting Information).

The 2D ¹⁵N-HSQC NMR spectrum of each positive hit was determined to further define the binding sites of the small molecule interactions with TDP-43 (Supplementary Figure 3 in the Supporting Information). Using TDP-43₁₋₂₆₀, small molecules were added at a 4:1 molar ratio. The addition of 5 out of 6 of the compounds either caused protein aggregation or showed little to no binding, possibly because of weak binding, and were not further characterized. However, the addition of nTRD022 (Figures 2A and 2B) caused visible

chemical shift perturbations but no protein aggregation and, hence, was chosen for further study.

In silico docking of nTRD22 predicted interactions with Glu3, Tyr43, Ser48, Gly59, and Ala66 (see Figure 2A). ^1H NMR of nTRD22 was used to evaluate the on-resonance energy transfer from STD-NMR of nTRD22 with TDP-43₁₋₂₆₀. The peaks seen in the STD-NMR spectrum were in the region of the benzyl group ((ii), 6.9–7.2 ppm) (see Figure 2C), confirming the Π -stacking interaction of the benzene group of nTRD22 with Tyr43 that was predicted by virtual screening. Microscale thermophoresis (MST) measured an apparent K_d value of $145 \pm 3 \mu\text{M}$ (Figure 2C) for TDP-43₁₋₂₆₀ and $96 \pm 36 \mu\text{M}$ for TDP-43₁₋₁₀₂ (NTD only) (Supplementary Figure 3 in the Supporting Information).

To further characterize where nTRD22 binds on TDP-43, we measured perturbations using a 2D ^{15}N -HSQC NMR with increasing concentrations of nTRD22, up to an 8:1 molar ratio of nTRD22:TDP-43₁₋₂₆₀ (Figures 2D-J). We observed chemical shifts in the 2D ^{15}N -HSQC of TDP-43₁₋₂₆₀, the majority of them occurring in the RRM region of the protein. To determine if these shifts were due to direct binding of nTRD22 to TDP-43₁₀₂₋₂₆₉ (RRM region), we collected a 2D ^{15}N -HSQC-NMR on TDP43₁₀₂₋₂₆₉ with nTRD22 at a 4:1 molar ratio (see Figures 2G and 2H). We observed no shifts in the spectra including the residues seen for the TDP-43₁₋₂₆₀ titration. nTRD22 was synthesized in-house for further experiments (see the Methods section in the Supporting Information) and was submitted to ^1H NMR and mass spectrometry for validation (see the supplementary figures provided in the Supporting Information).

To define which residues in the RRM domains were affected, we mapped the chemical-shift perturbations (CSPs) induced by nTRD22 onto the known structure of TDP-43₁₀₂₋₂₆₉ (PDB: 4bs2¹⁷) using a color gradient (Figures 2I and 2J). Interestingly, in addition to peak broadening, several of the shifted residues are involved in RNA binding. More precisely, Ile107 is part of the ribonucleotide interacting motif 1 and Lys145 is close to the ribonucleotide interacting motif 2, highly conserved short sequences known as RNP-1 (octameric sequence: KGFGFVRF in RRM1 and RAFAFVTF in RRM2) and RNP-2 (hexameric sequence: LIVLGL in RRM1 and VFVGRC in RRM2). Arg227 is part of RNP-1 in RRM2.¹⁸ Moreover, Cys173 is also shown to be affected in the presence of RNA.¹⁷ Replicates of this experiment showed a similar profile of residues affected by nTRD22 (Supplementary Figure 5 in the Supporting Information). Although the shifts are small, RNP-2 remains consistently shifted, even in different CSPs (Supplementary Figure 5); in addition, since RNP sequences are highly conserved sequence motifs on TDP-43 required for nucleic acid recognition, CSP data suggests that nTRD22 might indirectly modulate TDP-43 RNA binding. To test this, we used an amplified luminescent proximity homogeneous (alpha) assay recently developed for another compound targeting TDP-43¹⁵. TDP-43₁₋₂₆₀ binding to its canonical RNA sequence (UG₆) was measured with varying concentrations of compound and nTRD22 was able to inhibit 50% of the interaction with an IC_{50} value of $\sim 100 \mu\text{M}$ (Figure 2K). We also used surface plasmon resonance (SPR) to measure disruption of TDP-43-RNA binding (Figures 2L and 2M) and calculated an IC_{50} value of $\sim 145 \mu\text{M}$ and $\sim 30\%$ inhibition of TDP43-RNA interaction.

TDP-43 NTD can be responsible for dimer formation of the protein.¹¹ To test the effect of nTRD22 on TDP-43 dimerization, we immobilized NTD (TDP-43₁₋₁₀₂) on a CM5 chip and injection of TDP-43₁₋₂₆₀ (500 nM) resulted in binding (56 RU), because of self-interaction between TDP-43 NTD and TDP-43₁₋₂₆₀ (Supplementary Figure 6A in the Supporting Information). The experiment was repeated in the presence of 200 μ M nTRD22 or DMSO. Based on quantification of the response signals, there was no significant effect of nTRD22 on the interaction of TDP-43 NTD and TDP-43₁₋₂₆₀ (Supplementary Figure 6B in the Supporting Information). Thus, nTRD22 did not affect NTD dimerization.

Previous studies indicated that disruption of TDP-43 RNA binding in neuronal cells via the expression of RNA binding-deficient TDP-43 elicits the formation of intranuclear droplet-like structures and higher TDP-43 protein turnover.¹⁹ Most importantly, the authors showed neuroprotective effects of an RNA binding-deficient form of TDP-43. Because nTRD22 is able to reduce TDP-43 binding to RNA, we hypothesized that nTRD22 may be able to recapitulate some of the effects described with RNA binding-deficient TDP-43. To test this, we expressed TDP-43 fused to enhanced green fluorescent protein (TDP-43-EGFP) in rodent primary cortical neurons, applied nTRD22 at concentrations ranging from 5 μ M to 100 μ M, and imaged GFP positive cells by automated fluorescence microscopy.^{19,20} As expected, control cells incubated with dimethylsulfoxide (DMSO) exhibited a diffuse GFP signal, mainly in the nucleus, consistent with normal TDP-43 localization (Figure 3A). We observed the formation of intranuclear TDP-43-EGFP droplet-like structures in nTRD22 treated neurons, similar to those formed by an RNA binding-deficient variant TDP-43-EGFP¹⁹ (see Figures 3B and 3C, as well as Supplementary Figures 7A-7C in the Supporting Information). This was accompanied by a dose-dependent reduction in TDP-43-EGFP steady-state levels in treated neurons (Figure 3D). No change in the fluorescence of control cells transfected with EGFP was observed (Figure 3E). At higher doses, we noted toxicity upon application of nTRD22 to primary neurons (Supplementary Figure 7D in the Supporting Information), potentially because of interference with the endogenous TDP-43 function, other essential RNA binding proteins, or off-target effects. Consistent with the disruption experiments demonstrating the effects of nTRD22 on the RNA binding property of TDP-43 (Figures 2K-M), these data suggest that nTRD22 likely elicits TDP-43 phase separation and TDP-43 degradation by blocking RNA binding.

We also tested nTRD22 in a *Drosophila* line overexpressing human TDP-43, which is a well-known model of ALS.¹⁵ This model was shown to recapitulate important neuropathological and clinical features of the human proteinopathy, including TDP-43 aggregate formation, neuronal loss in an age-dependent manner, motor defects and reduced survival.²¹ Moreover, because of the advantages endowed by its small size, a short generation time, rapid propagation, and relatively small costs associated with stock maintenance, *Drosophila melanogaster* is an excellent animal model system to test compounds.

We used the climbing assay, which is a behavior that measures motor strength and coordination in adult flies. Briefly, when flies are placed on a vial, their innate behavior is to attempt to climb to the top of the vial, which is a behavior called negative geotaxis. A

sensitized version of the negative geotaxis assay that allows for earlier detection of milder defects over time was used according to ref 22 (see Figure 4A).

Flies expressing TDP-43 in motor neurons using the GAL4-UAS bipartite expression system exhibited locomotor defects in negative geotaxis (Figure 4 and Supplementary Figure 8 in the Supporting Information). nTRD22 was significantly able to rescue climbing defects compared to DMSO-treated or naive flies (Figure 4B). This rescue effect was increased in aged flies (see Supplementary Figure 8).

This study illustrates that the ability of nTRD22 to modulate TDP-43 RNA binding *in vitro* has neuroprotective properties in a *Drosophila* model of ALS. However, the exact mechanism by which the compound modulates TDP-43 binding to RNA is still unclear. We hypothesized that nTRD22 could influence residues important in RNA binding and allosterically modulate the RRM domain by (i) disrupting the binding of NTD to the RRM1 domain, leading to a modification of an open/closed conformation suggested in ref 23 or (ii) modifying the orientation of RRM1 and RRM2 toward each other, as previously discussed,¹⁹ or (iii) stabilizing a RNA-free form of TDP-43. The first hypothesis was tested by measuring chemical shift perturbations using 2D ¹⁵N-HSQC NMR. The NTD portion of TDP-43, residues 1–102, was added to TDP-43_{102–269} in a NTD: TDP-43_{102–269} molar ratio of 8:1. Data showed few significant perturbations related to NTD interacting with RRM domain (Supplementary Figure 9 in the Supporting Information). Hence, we do not think there is significant interaction between TDP-43 NTD and the RRM. Unless the loop region between NTD and the RRM domains influence binding, as this was not included in this experiment. Therefore, all hypotheses remain open to further investigation.

The compound nTRD22 offers new opportunities as a tool to further study allostery within TDP-43 and validate reduction of RNA binding by chemical modulation as a possible neuroprotective avenue. Furthermore, nTRD22, by its unique property of allosteric modulation, makes it possible to target TDP-43 with potentially less off-targeting of RRM domains, which are present in other RNA-binding proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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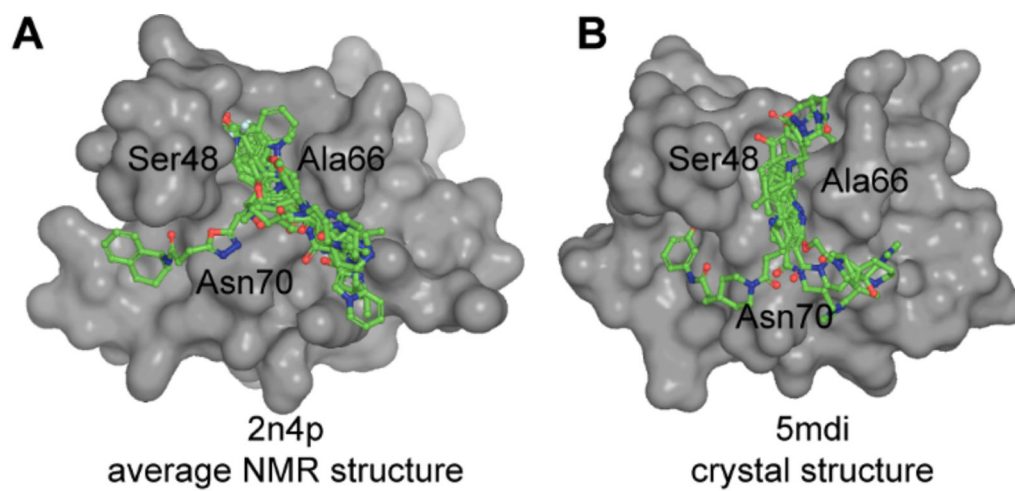


Figure 1. Docking of small molecules on N-terminal domain of TDP-43. Top 10 compounds (green, sticks and balls representation) from *in silico* docking on TDP-43-NTD: (A) NMR structure (PDB ID: 2n4p¹²) and (B) crystal structure (PDB ID: 5mdi⁸).

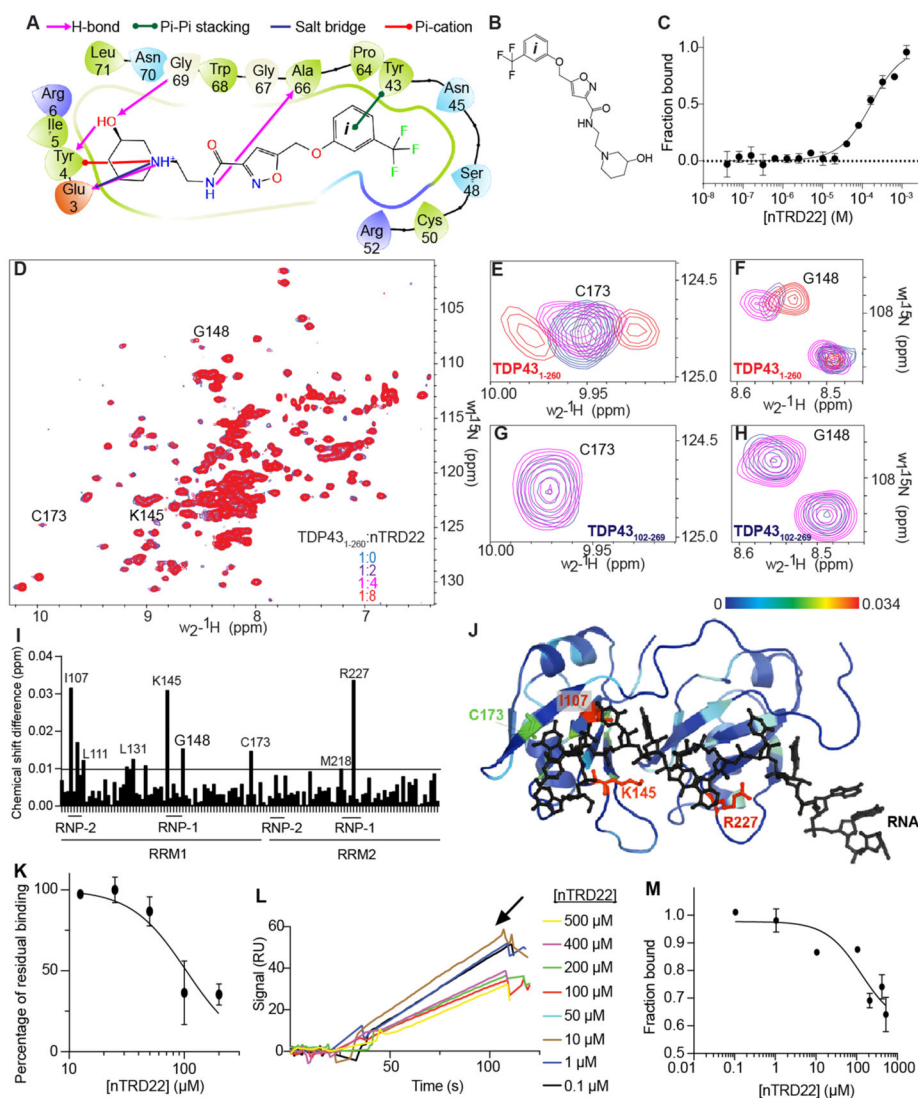


Figure 2. Biophysical characterization of nTRD22. (A) Two-dimensional (2D) representation of nTRD22 binding pocket in TDP-43 NTD obtained from Glide docking. nTRD22 is predicted to make (i) hydrogen bonds (shown in pink) with Glu3, Tyr4, Gly69, and Ala66, (ii) Π - Π stacking (shown in green) with Tyr43, (iii) a salt bridge (shown in blue) with Glu3, and (iv) a Π -cation interaction (shown in red) with Tyr4. (B) Structure of N-(2-(3-hydroxypiperidin-1-yl)ethyl)-5-((3-(trifluoromethyl)phenoxy)methyl)isoxazole-3-carboxamide (nTRD22). (C) MST values from thermographs of NT-647-labeled TDP43₁₋₂₆₀ in the presence of increasing concentrations (from 0.03 μ M to 1 mM) of nTRD22 were used to determine dissociation constant for binding of nTRD22 to TDP43₁₋₂₆₀. Apparent $K_d = 145 \pm 3 \mu$ M. Data are presented as mean \pm SD ($n = 3$). (D) Superposition of ^1H - ^{15}N heteronuclear single quantum correlation spectroscopy (HSQC) spectra of ^{15}N -labeled human TDP43₁₋₂₆₀ (100 μ M), free (blue) and in complex with nTRD22 with different ratios. (E-H) Close-up of shifts around TDP43 residues from TDP43₁₋₂₆₀ Cys173 (panel (E)) and Gly148 and Gly110 (panel (F))

or from TDP43₁₀₂₋₂₆₉ Cys173 (panel (G)) and Gly148 and Gly110 (panel (H)). (I) Chemical shift changes for assigned residues of the ¹⁵N-labeled TDP-43₁₋₂₆₀ (RRM portion only) upon complex formation with nTRD22. The average chemical shift changes of cross-peaks were calculated as described in the Methods section in the Supporting Information. The horizontal lines are thresholds (calculated as previously described¹⁷) above which a shift is considered significant with 2σ (back line). nTRD22 is able to induce shifts of residues localized in the ribonucleotide interacting motifs (RNP) of RRM1 and RRM2. (J) The chemical shift difference observed on TDP43₁₋₂₆₀ were mapped on TDP43₁₀₂₋₂₆₉ (PDB ID: 4bs2¹⁷). (K) A concentration-dependent curve was obtained for nTRD22's disruption of nucleic acid-TDP-43₁₋₂₆₀ interaction at a single RNA concentration (0.6 nM). Data are represented as mean \pm SEM ($n = 3$). (L) Representative SPR sensograms showing the signal for binding of 1 nM TDP-43₁₋₂₆₀ and immobilized (UG)₆ RNA in the presence of nTRD22 dissolved in DMSO (0.1–500 μ M). 150 RU of (UG)₆ RNA is immobilized on a SA chip. (M) A concentration-dependent curve was obtained for nTRD22's disruption of nucleic acid-TDP-43₁₋₂₆₀ interaction at a single RNA concentration showing $IC_{50} = 124 \mu$ M. Data are represented as mean \pm SEM ($n = 3$). Some error bars are smaller than the symbols.

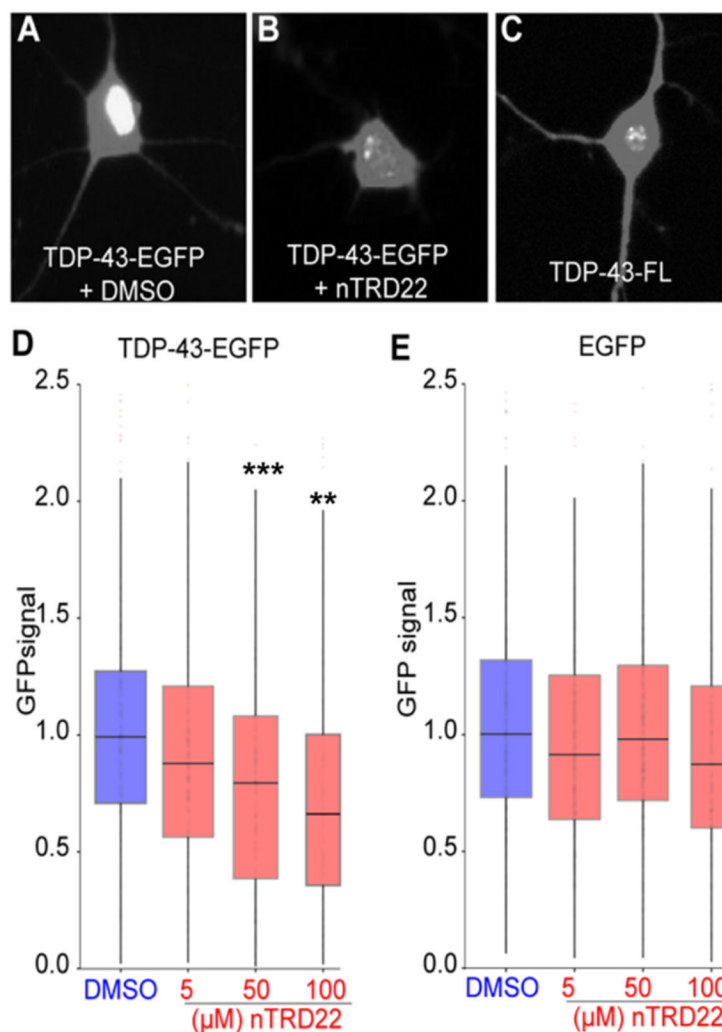


Figure 3. nTRD22 effect on primary cortical neurons. Confocal images of rodent primary cortical neurons with transfected TDP43-EGFP with (A) DMSO or (B) 100 μM nTRD22, or (C) transfected with TDP-43-FL (RNA binding-deficient mutant). Quantitation of GFP signal in TDP-43-EGFP (panel (D)) or EGFP (panel (E)) transfected primary neurons treated with increasing concentration of nTRD22 or DMSO. Data are presented as mean \pm SEM (n 183 neurons, from eight technical and three biological replicates). Statistical difference was assessed by using a Kruskal–Wallis test [legend: (***) $p < 0.001$; (**) $p = 0.01$].

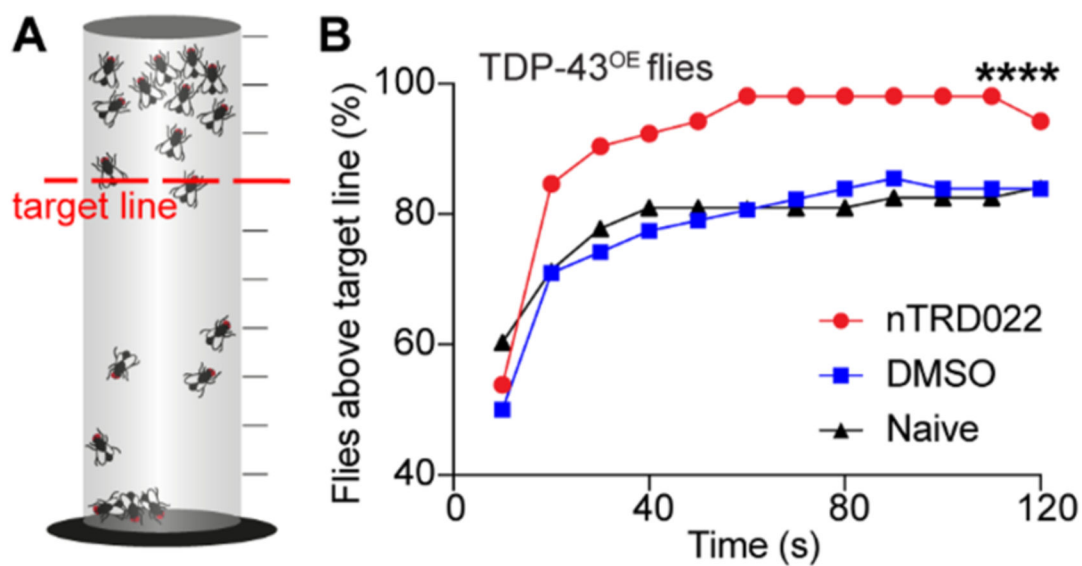


Figure 4. nTRD22 mitigates motor defects in a *Drosophila* model of ALS overexpressing TDP-43. (A) Principle of the sensitized version of the negative geotaxis assay.²² Flies are transferred without anesthesia to a wax-sealed glass graduated cylinder. Flies are tapped to the bottom, and their subsequent climbing activity is quantified for 2 min. The number of flies crossing the target line (red) at each time point chosen (every 10 s) is recorded. (B) Fly food supplemented with nTRD22 (50 μ M) resulted in increased motor performance, compared to naïve or DMSO-treated flies ($n = 60$, (****) $p < 0.0001$, two-way ANOVA).