Therapeutic potential of novel Cell Division Cycle Kinase 7 inhibitors on TDP-43-related pathogenesis such as Frontotemporal Lobar Degeneration (FTLD) and Amyotrophic Lateral Sclerosis (ALS)

Gabriela Vaca^{1*}†, Loreto Martinez-Gonzalez,^{2*} Ana Fernandez,¹ Elisa Rojas-Prats,² Gracia Porras,¹ Eva P. Cuevas², Carmen Gil,^{2,3} Ana Martinez^{2,3}, Ángeles Martín-Requero^{1,3}

¹Department of Molecular Biomedicine, Centro de Investigaciones Biológicas, Margarita Salas (CSIC), Ramiro de Maéztu 9, 28040 Madrid, Spain

²Department of Structural and Chemical Biology, Centro de Investigaciones Biológicas, Margarita Salas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain

³Centro de Investigación Biomédica en Red de Enfermedades neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, 28031 Madrid, Spain

* Equal contribution

† Present address: Escuela de Odontología, Facultad de Ciencias Medicas, Universidad Regional Autónoma de Los Andes UNIANDES, Ambato, EC180150, Ecuador

Running Title: CDC-7 inhibitors correct TDP-43 pathology in ALS and FLTD

Key words: FTLD-TDP, ALS, lymphoblasts, TDP-43, CDC-7 inhibitors

Address for correspondence:

Dr. Ángeles Martín-Requero, Dr. A. Martínez

Centro de Investigaciones Biológicas (CSIC)

Ramiro de Maeztu 9

28040 Madrid, SPAIN

Phone: 34-911098022

amrequero@cib.csic.es; ana.martinez@csic.es,

AM-R ORCID: 0000-0002-3416-9440

AM ORCID: 0000-0002-2707-8110

Abbreviations

ALS, amyotrophic lateral sclerosis
CDC-7, cell division cell cycle kinase-7
CDK6, cyclin-dependent kinase-8
CNS, central nervous system
C9orf72, chromosome 9 open reading frame 72
EBV, Epstein Barr virus
FTD, frontotemporal dementia
FTLD, frontotemporal lobar degeneration
GRN, granulin gene
LCLs, Lymphoblastoid cell lines
MAPT, microtubule-associated protein tau gene
PAMPA, parallel artificial membranes permeability assay
RRM, RNA-recognition motif
SOD1, superoxide dismutase 1
sALS, sporadic amyotrophic lateral sclerosis
TBK1, TANK-binding kinase 1
TDP-43, trans-activating response region DNA binding protein of 43 D
VCP, Valosin-Containing Protein

ABSTRACT

TDP-43 has been identified as the major component of protein aggregates found in affected neurons in FTLN-TDP and ALS patients. TDP-43 is hyperphosphorylated, ubiquitinated and cleaved in the C-terminus. CDC-7 was reported to phosphorylate TDP-43. There are not effective treatments for either FTLN-TDP or ALS, being a pressing need the search of new therapies. We hypothesized that modulating CDC-7 activity with small molecules able to interfere with TDP-43 phosphorylation could be a good therapeutic strategy for these diseases. Here, we have studied the effects of novel brain penetrant, thiopurine-based, CDC-7 inhibitors in TDP-43 homeostasis in immortalized lymphocytes from FTLN-TDP patients, carriers of a loss-of-function *GRN* mutation, as well as in cells derived from sporadic ALS patients. We found that selective CDC-7 inhibitors, ERP1.14a and ERP1.28a are able to decrease the enhanced TDP-43 phosphorylation in cells derived from FTLN-TDP and ALS patients and to prevent cytosolic accumulation of TDP-43. Moreover, treatment of FTLN-TDP lymphoblasts with CDC-7 inhibitors leads to recovering the nuclear function of TDP-43-inducing CDK6 repression. We suggest that CDC-7 inhibitors, mainly the heterocyclic compounds here shown, may be considered as promising drugs candidates for the ALS/FTD spectrum.

INTRODUCTION

Frontotemporal dementia (FTD) and Amyotrophic Lateral Sclerosis (ALS) are considered as extreme points of a disease spectrum on the basis of common genetic and neuropathological features (Couratier et al., 2017; Ling et al., 2013; Neumann, 2013). Among them, the presence of TDP-43 inclusions within the CNS is consistently found in both disorders (Lattante et al., 2015; Liscic, 2015).

ALS is a multisystem degenerative disorder characterized by the progressive loss of motoneurons, weakness of innervated muscles, and death by respiratory failure (Brooks, et al., 2000). On the other hand, FTD refers to a number of disorders with distinctive clinical phenotype caused by the loss of cortical neurons and basal ganglia, inducing changes in personality and language deficit (Hodges et al., 2004).

Although the clinical presentation of these diseases is different, there is a partial symptom overlap (Devenney et al., 2015). It has been estimated that about 10-15% of FTD patients develop some motor neuron impairment, and are then diagnosed with ALS, while about 15% of ALS patients develop cognitive and behavior problems similar to those observed in FTD patients (Lule et al., 2018; Phukan et al., 2012).

The identification of TDP-43 as the main component in protein deposits in ALS and FTD (Neumann et al., 2006) was followed by the discovery that mutations in the TAR DNA binding protein gene (*TARDBP*) (coding for TDP-43) is causative for both ALS and FTD (Sreedharan et al., 2008); (Kabashi et al., 2008) and later on by the identification of mutations in other genes such as *C9orf72*, *VCP* or *TBK1* could also cause ALS and FTD. Together these findings reinforce the idea of ALS and FTD are part of a disease spectrum (Burrell et al., 2016). However, genetic causes of isolated FTD or ALS might not cause ALS-FTD (Nguyen et al., 2018). For example, mutations in the *MAPT* gene (coding for tau protein) do not produce the characteristic TDP-43

pathology of ALS or ALS-FTD. Neither mutations in *SOD1* gene are associated with ALS-FTD cases (Canosa et al., 2014). Mutations in the *GRN* gene (coding for progranulin) are the second most common cause of familial FTD (Rohrer et al., 2009) but these rarely cause FTD-motor neuron disease, and *TARDBP* mutations are uncommon in FTD patients (Benajiba et al., 2009).

The characteristic pathological features in both ALS and FTD are selective neuronal loss, gliosis and cellular inclusions of TDP-43. TDP-43 is a 414-amino acid ubiquitously expressed nuclear protein, which contains two highly conserved RNA-recognition motifs (RRM1 and RRM2), a nuclear localization signal at the N-terminus, and a glycine-rich region mediating protein-protein interactions at the C-terminus (Lagier-Tourenne & Cleveland, 2009). Pathological C-terminal TDP-43 fragments of 25-35 KDa are ubiquitinated, hyperphosphorylated and accumulated as cellular inclusions in neurons and glial cells (Neumann et al., 2006). It is believed that abnormal phosphorylation of TDP-43 is a key event in the formation of toxic cellular aggregates (Nonaka et al., 2016). Several kinases appear to be involved in TDP-43 phosphorylation *in vitro* and *in vivo* (Hasegawa et al., 2008); (Liachko et al., 2013; Liachko et al., 2013, 2014; Moujalled et al., 2013), although it is unclear if they also contribute to mislocalization of the protein. Among these kinases Cell Division Cycle Kinase-7 (CDC-7) was shown to directly phosphorylate TDP-43 in cellular and animal models of TDP-43 proteinopathy (Liachko, et al., 2013). Therefore, the development of selective modulators of this kinase could be an interesting approach for therapeutic intervention in patients with ALS or FTD, particularly those patients with frontotemporal lobar degeneration (FTLD-TDP).

We have recently synthesized a focused chemical library of heterocyclic small molecules with inhibition of CDC-7 in the range of submicromolar and nanomolar range (Martínez et al., WO2018/172587, 2017). All of them are predicted to cross the human brain-blood barrier using a parallel artificial membranes permeability assay (PAMPA) methodology. The compounds are able to protect the cell line SH-SY5Y from the death caused by the increase of TDP-43 pathology (Rojas-Prats et al., Submitted). Here we report the effects of some of these compounds in rescuing TDP-43 homeostasis in immortalized lymphocytes from FTLTDP (Alquezar et al., 2016) or ALS patients (Posa et al., 2019).

METHODS

Materials.

RPMI 1640 culture medium Cat#: Lo500 was obtained from Biowest/Labclinics (Barcelona, Spain), Fetal Bovine Serum (FBS) Cat#: F7524 was purchased in (Merck, Madrid Spain). The CDC-7 inhibitors, ERPs were synthesized as previously described (WO2018/172587). The chemical structure, IC₅₀ values regarding CDC-7 inhibition together with effective permeability values, that predict their ability to cross the blood brain barrier (BBB) assessed by PAMPA (Di et al., 2003) are provided in Table 1. Antibodies against human TDP-43 (Cat#: 10782-2-AP) and phospho (409/410)-TDP-43 (Cat#: 22309-1AP) were obtained from Proteintech (Mancheser, UK). Antibodies against pRb (sc-50), CDK6 (sc-177), CDC-7(sc-56275), β -actin (sc-81178) and α -tubulin (sc-23948) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and anti-LaminB1 was purchased from Calbiochem (Billerica, MA, USA).

Study samples and cell lines

All participants in this study (listed in Table 2) were white caucasian. Five female patients, of Basque descent, were diagnosed in the Donostia hospital (San Sebastián, Spain) as FTLTDP, carrying a loss of function *GRN* mutation c.709 1-G>A. Five patients (two M/three F) were diagnosed as sporadic ALS in the Doce de Octubre hospital (Madrid, Spain) according to El Escorial criteria (Agosta et al., 2015). Mutations related to ALS and/or FTLTDP, including SOD1, C9ORF72, TARDBP and FUS, were excluded. Control healthy individuals were recruited separately and did not have any known neurological disorder.

All study protocols were approved by Donostia and Doce de Octubre (Approval number CEI 18/004) hospitals and the Spanish Council of Higher Research Institutional Review Board (date of approval Feb 1, 2018) and are in accordance with National and European Union Guidelines. The study was not pre-registered. Arrive guidelines were not applicable.

Peripheral blood samples were obtained after written informed consent of the patients or healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated on Lymphoprep™ (Cat#: 11508545) density-gradient centrifugation according to the instructions of the manufacturer (Axix-Shield Po CAS, Oslo, Norway). Cells were washed twice with phosphate-buffered saline, counted, and resuspended at the desired concentration. Establishment of lymphoblastoid cell lines (LCLs) was performed in our laboratory by infecting peripheral blood lymphocytes with the Epstein–Barr virus from the cell line B95.8 using cyclosporin A (Cat#: 59865-13-3), Sigma-Aldrich, (Madrid, Spain) to reduce the innate immune response and rejection of the virus essentially as described previously (Ibarreta et al., 1997). Cells were grown in suspension in T flasks in an upright position, in approximately 8 mL of RPMI-1640 medium that contained 2 mM L-glutamine, 100 µg/mL penicillin/streptomycin and, unless otherwise stated, 10% (v/v) FBS, and maintained in a humidified 5% CO₂ incubator at 37 °C. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium. In all experiments the different LCLs were used individually. Experiments were not blindly performed. The experimental outline is summarized in Fig. 1

Determination of cell proliferation.

Cell proliferation was determined by total cell counting, using a TC10™ Automated Cell Counter, Bio-Rad Laboratories, S.A. (Madrid, Spain). EBV-immortalized lymphocytes from control and *GRN* mutation carriers or sALS were seeded at an initial cell concentration of 1×10^6 cells $\times$ mL⁻¹ and enumerated 72 h later. Cells failing to exclude the dye were considered nonviable.

Immunoblotting analysis.

Cells were collected by centrifugation, washed with PBS and total protein extracts were obtained by lysing them as previously described (Alquezar et al., 2016). Nuclear and cytosolic fractions were obtained using the Subcellular Protein Fractionation Kit, (Cat#: 78840, Thermo Fisher Scientific, Madrid, Spain) following the manufacturer's instructions. Lamin B and α -Tubulin were used as markers for nuclear and cytosolic fractions respectively. The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Cat#: 23225, ThermoFisher, Madrid Spain). Equal amounts of proteins were resolved by SDS–polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes and immunodetected, as previously described (Alquezar et al., 2014). The following primary antibodies were used, TDP-43 (1:1000); phospho-(S409/410)-TDP-43 (1:500); β -actin (1:500); α -tubulin (1:1000) and Lamin B1 (1:1000), pRb (1:500), CDK6 (1:1000), and CDC-7 (1:500) Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Cat#: 1662408EDU, Bio-Rad, Alcobendas, Madrid, Spain) and detected with a chemiluminiscent substrate detection system ECL (Cat#: W1001, Promega Biotech Ibérica, SL, Alcobendas, Madrid, Spain).

Relative band intensities were quantified using Image Studio Lite software (LI-COR Biotechnology, NE, USA).

Immunofluorescence

Subcellular localization of TDP-43 was assessed in human lymphoblasts by immunofluorescence. Cells (1×10^6 ml⁻¹) were incubated in RPMI medium containing 10% FBS for 24 h and then fixed for 30 min in 4% paraformaldehyde in PBS and blocked and permeabilized with 0.5% TritonX-100 in PBS-0.5% BSA for 60 min at room temperature. Cells were attached to poly-L-lysine coated coverslips using the Cytospin centrifuge at 700 rpm for 10 minutes and then incubated overnight with the anti-TDP43 polyclonal antibody. After removing the primary antibody, cells were washed with PBS and were incubated with Alexa Fluor 488-conjugated anti-rabbit antibody (Cat#: A0545, Sigma -Aldrich, Madrid, Spain). Preparations were mounted on ProLong® Gold Antifade Reagent with DAPI (Cat#: P36931, Thermo Fisher) allowing nuclear visualization. High-resolution images were acquired for ~30 cells per group in two independent experiments using a confocal microscope Zeiss 510 equipped with a META detection system and a 63× oil immersion objective.

Quantitative real-time PCR

Total RNA was extracted from cell cultures using Trizol reagent (Cat: AM9738 Thermo Fisher, Alcobendas, Madrid, Spain). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to determine the integrity of 18S and 28S ribosomal RNA. RNA was then treated with DNase I Amplification Grade (Cat#: 1806815, Invitrogen, Alcobendas, Madrid, Spain). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Cat#: 10432122, Invitrogen, Alcobendas,

Madrid, Spain). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicates using TaqMan Universal PCR MasterMix No AmpErase UNG (Cat#: 10167154, Applied Biosystems, Alcobendas, Madrid, Spain) reagent according to the manufacturer's protocol. Primers were designed using the Universal ProbeLibrary for Human (Roche Applied Science, Madrid, Spain) and used at a final concentration of 20 μ M. The sequences of the forward and reverse primers used are the following: for CDK6 5'-tgatcaactaggaataatcttggac-3' and 5'-ggcaacatctctaggccagt-3'; for β -actin, 5'-ccaaccgcgagaagatga-3' and 5'-ccagaggcgtacagggatag-3'. Real time quantitative PCR was performed in the Bio-Rad iQ5 system using a thermal profile of an initial 5-minute melting step at 95 °C followed by 40 cycles at 95 °C for 10 seconds and 60 °C for 60 seconds. Relative messenger RNA (mRNA) levels of the genes of interest, (RNA sequences can be found, in <http://www.ncbi.nlm.nih.gov/GenBank/index.html>), were normalized to β -actin expression using the simplified comparative threshold cycle delta-delta CT method ($2^{-[\Delta\text{CT CDK6} - \Delta\text{CT Actin}]}$).

Statistical analysis

Statistical analyses were performed with Graph Pad Prism 8 (La Jolla, CA, USA). All the statistical data are presented as mean $\pm$ standard error of the mean (SEM). Since a Shapiro-Wilk test showed normal distribution of all data sets in this study, a parametric analysis of variance (ANOVA) followed by the Fisher test for multiple comparisons was applied to test for statistical differences of the means. A value of $p < 0.05$ was considered significant. No statistical test for outliers was conducted and no data point were excluded. No official randomization method was used

RESULTS

Common and distinct features of lymphoblasts from FTLD-TDP and ALS patients.

We recently reported that immortalized lymphocytes from FTLD-TDP (Alquezar et al., 2016) and ALS patients (Posa et al., 2019) recapitulate the main pathological changes in TDP-43 homeostasis observed in the affected neurons, such as increased levels of phosphorylation and accumulation of TDP-43 in the cytosolic compartment. A comparative analysis of postransductional changes in TDP-43 (phosphorylation and truncation), as well as subcellular distribution of TDP-43, in lymphoblasts derived from control and individuals diagnosed with FTLD-TDP, carriers of a loss-of-function *GRN* mutation, and sporadic ALS patients shows, in agreement with previous reports, an increase in the levels of phosphorylated full length and truncated TDP-43 protein in cell derived from ALS or FTLD-TDP patients (Fig. 2A). Moreover, cytosolic TDP-43 accumulation is appreciated in both FTLD-TDP and ALS lymphoblasts (Fig. 2B). These alterations in TDP-43 homeostasis were accompanied by increased proliferative activity as well as higher levels of CDK6 (mRNA and protein), and phosphorylated pRb in lymphoblasts derived from FTLD-TDP patients, while no change in proliferation and CDK6/pRB content were observed in ALS lymphoblasts when compared with control cells (Fig. 2C).

CDC-7 is located in cellular cytoplasm.

CDC-7 is known to phosphorylate TDP-43 *in vitro*. However, since main CDC-7 function is to promote initiation and maintenance of DNA replication within the nucleus (Kim et al., 2003), we wonder if there is a significant nucleo-cytoplasmic shuttling of

this kinase that could explain cytosolic TDP-43 phosphorylation in ALS or FTLD-TDP lymphoblasts. For these reasons, we determined the total protein levels as well as performed cell extracts fractionation experiments to study subcellular CDC-7 localization in lymphoblasts from control individuals and patients. As shown in Fig. 3A a significant increase in total CDC-7 levels is apparent in FTLD-TDP and ALS patients. Interestingly, we found significant cytosolic levels of CDC-7 levels, where presumably TDP-43 pathology occurs, in human lymphoblasts (Fig. 3B).

CDC-7 inhibitors decrease TDP-43 phosphorylation in lymphoblasts from patients from FTLD-TDP and ALS patients.

Next, we evaluated the effects of several in-house CDC-7 inhibitors on the levels and phosphorylation status of TDP-43 in control and FTD and ALS patients. TDP-43 phosphorylation was assessed by Western blotting using a phospho-specific (Ser 409/410) anti-TDP-43 antibody, while cellular levels of TDP-43 were detected with an anti N-terminal TDP-43 antibody. As expected, lymphoblasts from either FTLD-TDP or ALS patients showed higher levels of phosphorylated TDP-43 compared with control cells (Fig. 4A,B). Treatment of patients cells with several selective inhibitors of CDC-7 for 24 h, diminished levels of p-TDP-43, reaching values close to those observed in control cells (Fig. 4A,B). These compounds did not affect control levels of p-TDP-43, neither affect the non-phosphorylated TDP-43 content in control and ALS or FTLD-TDP patients (Fig. 4A,B). We selected two of these compounds, named ERP1.14a and ERP1.28a, to further characterize their cellular effects in control, FLTD-TDP and ALS lymphoblasts.

Effects of CDC-7 inhibitors on subcellular localization of TDP-43 in lymphoblasts from FTLD-TDP and ALS patients.

Next, we evaluated the effects of ERP1.14a and ERP1.28a, in subcellular localization of TDP-43 in control or FTLD-TDP lymphoblasts. Fig. 5A,B shows the results of nuclear and cytoplasmic fractionation experiments. It is shown that TDP-43 accumulated in the cytosolic compartment of FTLD-TDP lymphoblasts (Fig. 5A), while levels of nuclear TDP-43 decreased in comparison with control cells (Fig. 5B). CDC-7 inhibitors had no appreciable effects on control cells, but were able to significantly decrease the accumulation of cytosolic TDP-43 in FTLD-TDP lymphoblasts, observing a trend to restore normal nuclear levels of this protein, although changes did not reach statistical significance (Fig. 5A,B). Similar results were obtained after treating ALS lymphoblasts with ERP1.14a and ERP1.28 a (Fig. 6A,B).

Effects of CDC-7 inhibitors on TDP-43 functionality

It is known that TDP-43 repress *CDK6* gene expression (Ayala et al. 2008). Indeed, we previously demonstrated in *GRN* mutated lymphoblast, upregulation of CDK6 secondary to decreased TDP-43 nuclear content (Alquezar et al., 2012a). For these reasons, we have evaluated the effects of ERP1.14a and ERP1.28a on both *CDK6* mRNA and protein levels of CDK6. Real-time PCR analyses confirmed the previously reported increased levels of CDK6 levels in lymphoblasts from FTLD-TDP patients (Alquezar et al., 2012b; Alquezar et al., 2016) (Fig. 7A) and demonstrated that inhibiting TDP-43 phosphorylation by targeting CDC-7 kinase is sufficient to restore the levels of mRNA CDK6 and protein to control values (Fig. 7A,B). In contrast, we did not find significant changes in CDK6 levels in lymphoblasts from ALS patients (Fig. 7A,B). Together these results may explain the differences in proliferative activity between progranulin-deficient lymphoblasts and ALS cells (Fig. 7C). Treatment of FTLD-TDP lymphoblasts with ERP1.14a or ERP1.28a restored normal rates of cell proliferation.

DISCUSSION

FTLD-TDP and ALS are two neurodegenerative disorders that overlap in their clinical presentation, genetics and pathology. To date there are not effective disease-modifying treatment for FTD and/or ALS, which remain incurable.

TDP-43 proteinopathy is a hallmark for the ALS/FTD spectrum. Increasing knowledge of pathological processing of TDP-43 opened new avenues for the identification of novel targets and the discovery of effective drugs (Palomo et al., 2019). Efforts had been towards the modulation of pathological posttranslational modifications of TDP-43, rather than in the control of protein expression levels (Buratti, 2018). Prominent modifications of TDP-43 associated with neurodegeneration are hyperphosphorylation of the molecule in Ser 409/410 residues, formation of C-terminal fragments and mislocalization and cytoplasmic accumulation of TDP-43 (Neumann et al., 2006); (Heyburn & Moussa, 2017).

We present here evidences for the role of CDC-7 kinase in pathological hyperphosphorylation of TDP-43 in cells derived from FTLD-TDP patients, carriers of a *GRN* mutation, as well as in sporadic ALS patients. Recently we have developed a serie of potent CDC-7 inhibitors able to cross the BBB barrier (Rojas-Prats et al., submitted). Here we show the efficacy of some of these of CDC-7 inhibitors in restoring the pathological TDP-43 modifications in lymphoblasts from FTLD-TDP and ALS patients.

We have used lymphoblastoid cell lines from FTLD-TDP and ALS patients, based in previous reports from this laboratory indicating that these cell lines recapitulate main pathological hallmarks of both FTLD-TDP and ALS, showing increased TDP-43 phosphorylation and cytoplasmic accumulation of the protein (Alquezar et al., 2016;

Posa et al., 2019). Therefore, we considered these cell lines a useful human-based platform for preclinical studies with new pharmacological agents.

Our results indicated an apparent increase in both cytosolic and nuclear levels of CDC-7 kinase in lymphoblasts from FTLD-TDP and ALS patients as compared with protein levels in control cells, which may point to the involvement of CDC-7 in ALS and FTLD-TDP pathology. Moreover, the abundance of CDC-7 in the cytoplasm suggests non-nuclear functions for this kinase. Therefore, it is possible that increased CDC-7 activity in FTD/ALS could lead to the enhanced TDP-43 phosphorylation in disease-affected cells. The observation that CDC-7 colocalize with phosphorylated TDP-43 in the frontal cortex of cases of FTLD-TDP supports a role of this kinase in pathological TDP-43 phosphorylation *in vivo* (Liachko et al., 2013).

TDP-43 phosphorylation and protein mislocalization are equally affected in progranulin deficient-FTLD-TDP and ALS patients derived cells. The novel CDC-7 inhibitors were able to prevent the enhanced TDP-43 phosphorylation in either FTLD-TDP or ALS lymphoblasts. Moreover, the compounds ERP1-14a and ERP1.28a were able not only to correct increased TDP-43 phosphorylation, but also to recover the nuclear localization of TDP-43, providing additional support to the idea that these two events are interconnected (Choksi et al., 2014; Alquezar et al., 2016; Prasad et al., 2019). It is worth mentioning, that although the effect of CDC-7 inhibitors on TDP-43 homeostasis in LCLs lines from non *GRN*-linked FTLD-TDP patients had not been tested, most likely, ERP1.14a and ERP1.28a could also be effective in modulating TDP-43 phosphorylation in a progranulin levels-independent manner, in those patients, taking into account the probed efficacy of these compounds in cellular and murine models of TDP-43 proteinopathy in which the levels of progranulin remain constant (Rojas-Prats et al., submitted).

TDP-43 is known to play a diverse array of functions, most of them related to RNA metabolism. The fundamental question of whether TDP-43 dysfunction mediates neurodegeneration through loss of normal nuclear functions or by a gain of toxic action in the cytoplasm is not solved yet.

We and others suggested that decreased nuclear TDP-43 levels in a number of cell types resulted in enhanced CDK6/pRb pathway (Ayala et al., 2008; Alquezar et al., 2012b). TDP-43 binds UG repeats within CDK6 sequence to repress mRNA CDK6 expression (Ayala et al., 2008). We had previously reported that increased phosphorylation and mislocalization of TDP-43 in immortalized lymphoblasts from FTLD-TDP patients results in increased proliferative activity when compared with cells from healthy donors (Alquezar et al., 2012b; Alquezar et al., 2016). Our results indicated that modulation of TDP-43 phosphorylation by targeting CDC-7 kinase is sufficient, not only to rescue nuclear TDP-43 localization and to decrease mRNA *CDK6* and protein levels in lymphoblasts from FTLD-TDP patients, but also to normalize rates of cell proliferation. In contrast, no change in both mRNA CDK6 and protein levels were observed in ALS lymphoblasts when compared to control cells, despite the decrease in nuclear TDP-43 levels. These findings may explain the differences in proliferative activity of lymphoblasts from FTLD-TDP or ALS patients. Considering that the functional consequences of TDP-43 hyperphosphorylation and nuclear depletion may affect different cellular pathways, it is important to obtain a full understanding of the precise TDP-43-controlled pathways responsible for specific disease onset and progression.

In summary, the present results suggest the involvement of CDC-7 kinase in pathological phosphorylation and mislocalization of TDP-43 in cells derived from ALS and FTLD patients. We show here the ability of the novel thiopurine-based CDC-7 inhibitors, to recover TDP-43 homeostasis in either ALS or FTLD-TDP lymphoblast.

We, therefore suggest that CDC-7 inhibitors, mainly the heterocyclic compounds here shown may be considered as promising drugs candidates for the ALS/FTD spectrum.

CONCLUSIONS

Targeting CDC-7 kinase with small molecules could be potentially useful in treatment of FTLD-TDP and ALS pathologies. The novel brain permeable selective CDC-7 inhibitors, ERP1.14a and ERP1.28a are able to reduce TDP-43 phosphorylation, and even more important, to rescue aberrant cytoplasmic TDP-43 accumulation in both FTLD-TDP and ALS lymphoblasts. Moreover, treatment of FTLD-TDP lymphoblasts with ERP1.14a or ERP1.28a leads to recovering the nuclear function of TDP-43 on promoting CDK6 repression. Therefore, these compounds can be considering promising drug candidates for the future pharmacotherapy of TDP-43 proteinopathies.

ACKNOWLEDGEMENTS

This work has been supported by grants from Comunidad de Madrid (ELA_Madrid grant B2017/BMD-3813), from the Ministerio de Ciencia, Innovación y Universidades (SAF2016-76693-R to A.M), and ISCiii CIBERNED (CB18/05/00040). G.V. was supported by a fellowship from SENECYT (Ecuador). E.R. was supported by FPU14-00204 (Ministerio de Educación, Ciencia y Deporte). The authors would like to thank Drs. A. G. Redondo, J. Esteban (Hospital Doce de Octubre) and Dr. López de Munain (Hospital Donostia) as well as to all patients and their families involved in this study.

REFERENCES

- Agosta F, Al-Chalabi A, Filippi M, Hardiman O, Kaji R, Meininger V, *et al.* (2015). The El Escorial criteria: strengths and weaknesses. *Amyotrophic lateral sclerosis & frontotemporal degeneration* 16, 1-7.
- Alquezar C, Esteras N, Bartolome F, Merino JJ, Alzualde A, de Munain AL, *et al.* (2012a). Alteration in cell cycle-related proteins in lymphoblasts from carriers of the c.709-1G>A PGRN mutation associated with FTLT-TDP dementia. *Neurobiology of aging* 33, 429.e427-420.
- Alquezar C, Esteras N, Alzualde A, Moreno F, Ayuso MS, Lopez de Munain A, *et al.* (2012b). Inactivation of CDK/pRb pathway normalizes survival pattern of lymphoblasts expressing the FTLT-Progranulin mutation c.709-1G>. *PLoS one*, 7(5):e37057
- Alquezar C, Esteras N, de la Encarnacion A, Alzualde A, Moreno F, Lopez de Munain A, *et al.* (2014). PGRN haploinsufficiency increased Wnt5a signaling in peripheral cells from frontotemporal lobar degeneration-progranulin mutation carriers. *Neurobiol Aging* 35, 886-898.
- Alquezar C, Salado IG, de la Encarnacion A, Perez DI, Moreno F, Gil C, *et al.* (2016). Targeting TDP-43 phosphorylation by Casein Kinase-1delta inhibitors: a novel strategy for the treatment of frontotemporal dementia. *Mol Neurodegener* 11, 36.
- Ayala YM, Misteli T, & Baralle FE (2008). TDP-43 regulates retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression. *Proceedings of the National Academy of Sciences of the United States of America* 105, 3785-3789.

Benajiba L, Le Ber I, Camuzat A, Lacoste M, Thomas-Anterion C, Couratier P, *et al.* (2009). TARDBP mutations in motoneuron disease with frontotemporal lobar degeneration. *Annals of neurology* 65, 470-473.

Brooks BR, Miller RG, Swash M, & Munsat TL (2000). El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 1, 293-299.

Buratti E (2018). TDP-43 post-translational modifications in health and disease. *Expert opinion on therapeutic targets* 22, 279-293.

Burrell JR, Halliday GM, Kril JJ, Ittner LM, Gotz J, Kiernan MC, *et al.* (2016). The frontotemporal dementia-motor neuron disease continuum. *Lancet* 388, 919-931.

Canosa A, Calvo A, Moglia C, Iazzolino B, Brunetti M, Restagno G, *et al.* (2014). A familial ALS case carrying a novel p.G147C SOD1 heterozygous missense mutation with non-executive cognitive impairment. *Journal of neurology, neurosurgery, and psychiatry* 85, 1437-1439.

Choksi DK, Roy B, Chatterjee S, Yusuff T, Bakhoun MF, Sengupta U, *et al.* (2014). TDP-43 Phosphorylation by casein kinase Iepsilon promotes oligomerization and enhances toxicity in vivo. *Human molecular genetics* 23, 1025-1035.

Couratier P, Corcia P, Lautrette G, Nicol M, & Marin B (2017). ALS and frontotemporal dementia belong to a common disease spectrum. *Revue neurologique* 173, 273-279.

Devenney E, Vucic S, Hodges JR, & Kiernan MC (2015). Motor neuron disease-frontotemporal dementia: a clinical continuum. *Expert review of neurotherapeutics* 15, 509-522.

Di L, Kerns EH, Fan K, McConnell OJ, & Carter GT (2003). High throughput artificial membrane permeability assay for blood-brain barrier. *European journal of medicinal chemistry* 38, 223-232.

Hasegawa M, Arai T, Nonaka T, Kametani F, Yoshida M, Hashizume Y, *et al.* (2008). Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Ann Neurol* 64, 60-70.

Heyburn L, & Moussa CE (2017). TDP-43 in the spectrum of MND-FTLD pathologies. *Molecular and cellular neurosciences* 83, 46-54.

Hodges JR, Davies RR, Xuereb JH, Casey B, Broe M, Bak TH, *et al.* (2004). Clinicopathological correlates in frontotemporal dementia. *Annals of neurology* 56, 399-406.

Ibarreta D, Parrilla R, & Ayuso MS (1997). Altered Ca²⁺ homeostasis in lymphoblasts from patients with late-onset Alzheimer disease. *Alzheimer Dis Assoc Disord* 11, 220-227.

Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Vande Velde C, *et al.* (2008). TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nature genetics* 40, 572-574.

Kim JM, Yamada M, & Masai H (2003). Functions of mammalian Cdc7 kinase in initiation/monitoring of DNA replication and development. *Mutation research* 532, 29-40.

Lagier-Tourenne C, & Cleveland DW (2009). Rethinking ALS: the FUS about TDP-43. *Cell* 136, 1001-1004.

Lattante S, Ciura S, Rouleau GA, & Kabashi E (2015). Defining the genetic connection linking amyotrophic lateral sclerosis (ALS) with frontotemporal dementia (FTD). *Trends in genetics : TIG* 31, 263-273.

Liachko NF, McMillan PJ, Guthrie CR, Bird TD, Leverenz JB, & Kraemer BC (2013). CDC7 inhibition blocks pathological TDP-43 phosphorylation and neurodegeneration. *Annals of neurology* 74, 39-52.

Liachko NF, McMillan PJ, Strovast TJ, Loomis E, Greenup L, Murrell JR, *et al.* (2014). The tau tubulin kinases TTBK1/2 promote accumulation of pathological TDP-43. *PLoS genetics* 10, e1004803.

Ling SC, Polymenidou M, & Cleveland DW (2013). Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron* 79, 416-438.

Liscic RM (2015). Molecular basis of ALS and FTD: implications for translational studies. *Arhiv za higijenu rada i toksikologiju* 66, 285-290.

Lule D, Bohm S, Muller HP, Aho-Ozhan H, Keller J, Gorges M, *et al.* (2018). Cognitive phenotypes of sequential staging in amyotrophic lateral sclerosis. *Cortex; a journal devoted to the study of the nervous system and behavior* 101, 163-171.

Martínez A, Pérez DI, Gil C, Martín-Requero A, Rojas-Prats E, Martínez-González L, Pérez. C, CDC7-inhibitor compounds and use thereof for the treatment of neurological conditions. WO2018/172587, March 22, 2017.

Moujalled D, James JL, Parker SJ, Lidgerwood GE, Duncan C, Meyerowitz J, *et al.* (2013). Kinase Inhibitor Screening Identifies Cyclin-Dependent Kinases and Glycogen Synthase Kinase 3 as Potential Modulators of TDP-43 Cytosolic Accumulation during Cell Stress. *PloS one* 8, e67433.

Neumann M (2013). Frontotemporal lobar degeneration and amyotrophic lateral sclerosis: molecular similarities and differences. *Revue neurologique* 169: 793-798.

Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, *et al.* (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 314, 130-133.

Nguyen HP, Van Broeckhoven C, & van der Zee J (2018). ALS Genes in the Genomic Era and their Implications for FTD. *Trends in genetics* : TIG 34, 404-423.

Nonaka T, Suzuki G, Tanaka Y, Kametani F, Hirai S, Okado H, *et al.* (2016). Phosphorylation of TAR DNA-binding Protein of 43 kDa (TDP-43) by Truncated Casein Kinase 1delta Triggers Mislocalization and Accumulation of TDP-43. *J Biol Chem* 291, 5473-5483.

Palomo V, Tosat-Bitrian C, Nozal V, Nagaraj S, Martin-Requero A, & Martinez A (2019). TDP-43, A Key Therapeutic Target beyond Amyotrophic Lateral Sclerosis. *ACS chemical neuroscience* 10: 1183-1196.

Phukan J, Elamin M, Bede P, Jordan N, Gallagher L, Byrne S, *et al.* (2012). The syndrome of cognitive impairment in amyotrophic lateral sclerosis: a population-based study. *Journal of neurology, neurosurgery, and psychiatry* 83, 102-108.

Posa D, Martinez-Gonzalez L, Bartolome F, Nagaraj S, Porras G, Martinez A, *et al.* (2019). Recapitulation of Pathological TDP-43 Features in Immortalized Lymphocytes from Sporadic ALS Patients. *Molecular neurobiology* 56, 2424-2432.

Prasad A, Bharathi V, Sivalingam V, Girdhar A, & Patel BK (2019). Molecular Mechanisms of TDP-43 Misfolding and Pathology in Amyotrophic Lateral Sclerosis. *Frontiers in molecular neuroscience* 12, 25.

Rohrer JD, Guerreiro R, Vandrovcsa J, Uphill J, Reiman D, Beck J, *et al.* (2009). The heritability and genetics of frontotemporal lobar degeneration. *Neurology* 73, 1451-1456.

Rojas-Prats E, Martinez-Gonzalez L, Liachko NF, Gonzalo C, Pérez C, Petralla S, Monti B, Kraemer BC, Martin-Requero A, Gil C, Perez, DI, De Lago, E and Martinez A. Targeting nuclear protein TDP-43 by Cell division cycle kinase 7 inhibitors: a new therapeutic approach for amyotrophic lateral sclerosis (Submitted).

Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, *et al.* (2008). TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* (New York, NY) 319, 1668-1672.

Table 1. Overview of the compounds used in this study

Compound Name	Chemical structure	CDC-7 IC ₅₀ μ M	Pe 10 ⁻⁶ cm.s ⁻¹	BBB prediction
ERP1.14a		0.125	6.2 $\pm$ 1.1	CNS+
ERP1.17a		0.340	13.2 $\pm$ 2.0	CNS+
ERP1.28a		0.140	15.1 $\pm$ 0.6	CNS+
ERP2.37		0.270	13.1 $\pm$ 0.1	CNS+

IC₅₀: compound concentration able to inhibit the 50% of human recombinant CDC-7 activity *in vitro*. Pe: apparent permeability by PAMPA assay. Compounds were classified as CNS+ (brain penetrant) when permeability is $> 3.74 \cdot 10^{-6}$ cm.s⁻¹. Values shown are the mean $\pm$ SD

Table 2. Clinical features of healthy controls, and FTLD-TDP and ALS patients

Patient ID	Sex	Age	Diagnosis	Mutation	Cognitive impairment
C1	F	57			
C2	M	55			
C3	F	67			
C4	F	58			
C5	M	75			
FTLD-TDP-1	F	70	FTLD-TDP	<i>GRN</i> c.709-1G>A	yes
FTLD-TDP-2	F	70	FTLD-TDP	<i>GRN</i> c.709-1G>A	yes
FTLD-TDP-3	F	68	FTLD-TDP	<i>GRN</i> c.709-1G>A	yes
FTLD-TDP-4	F	60	FTLD-TDP	<i>GRN</i> c.709-1G>A	yes
FTLD-TDP-5	F	54	FTLD-TDP	<i>GRN</i> c.709-1G>A	yes
ALS-1	F	76	sALS	ND*	no
ALS-2	F	54	sALS	ND	no
ALS-3	M	54	sALS	ND	no
ALS-4	M	79	sALS	ND	no
ALS-5	M	68	sALS	ND	no

*negative for *SOD-1*, *TARBP*, and *C9orf72*

Legends to the Figures

Figure 1.- Experimental design flow-chart.

Type (FTLD, control, ALS) and number (n) of lymphoblasts human samples are depicted. Full description of each individual sample is collected in Table 1. Number of different CDC-7 inhibitors used in the study is also noted

Figure 2.- TDP-43 homeostasis, CDK6/pRb pathway, and proliferative activity of control, FTLD-TDP, and ALS lymphoblasts.

Immortalized lymphocytes from control (n=5), and c.709-1G>A *GRN* mutation carriers (n=5), and ALS patients (n=5), were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ and cultured in RPMI medium containing 10% FBS for 72 h. A) Cells were harvested and processed for Western blotting analysis 24 h of cell seeding, to evaluate the phosphorylation status of TDP-43. A representative immunoblot is shown. B) TDP-43 protein localization was assessed by confocal laser scanning microscopy. Cells were stained with anti-TDP-43 antibody followed by secondary antibody labeled with Alexa Fluor 488. DAPI was included in the mounting media to stain the nucleus. Merged images show that FTLD-TDP and ALS cells present higher cytosolic localization of TPD-43 protein. C) Cells were harvested, 24 h after seeding, to isolate RNA and to prepare cell lysates. *CDK6* mRNA expression levels were analyzed by quantitative RT-PCR (left panel). Representative immunoblots showing increased pRb phosphorylation in FTLD-TDP lymphoblasts compared with control or ALS cells are shown (central panel). Proliferation activity was determined by cell counting 72 h after serum stimulation. Horizontal bars represent the mean of the proliferative activity of five LCLs on each group (right panel)

Figure 3.- CDC-7 is expressed in both cytosol and nucleus in human lymphoblast.

Control, FTLT-DTP, and ALS lymphoblasts were seeded as described in the legend to Fig.2. 24 h latter, cells were collected and lysed to obtain either total protein extracts (A), or fractionated cytosolic and nuclear extracts (B), that were analyzed by Western blotting. Densitometric analyses represent the mean $\pm$ SEM of different observations carried out in different cell lines (n=5) from each group (*p<0.05 significantly different from control cells. B) A representative experiment shows that CDC-7 is located in cytosol and nucleus. α -tubulin and LaminB1 antibodies were used as loading and purity control of the cytosolic and nuclear fractions respectively.

Figure 4.- Effects of novel CDC-7 inhibitors on phosphorylation of TDP-43 in control, FTLT-DTP and ALS lymphoblasts.

Control, FTLT-DTP, and ALS lymphoblasts were seeded as described in the legend to Fig.1 in the absence and in the presence of ERP1.14a, ERP1.17a, ERP1.28a and ERP2.37 (10 μ M). 24 hours after drugs addition, cells were harvested and processed for Western blotting analysis. The image represents an immunoblot showing the effect of CDC7 inhibitors decreasing the phosphorylation status of TDP-43 in FTLT-DTP lymphoblasts (A) or ALS lymphoblasts (B). The plots bellows represent the quantifications of the 43 KD pTDP-43 band normalized by Total-TDP-43. The densitometric analyses represent the mean $\pm$ SEM of different observations carried out in cell lines (n=5) from each group (**p<0.001 significantly different from control cells. †p<0.05, ††p<0.01, and †††p<0.001 significantly different from untreated cells).

Figure 5.- Effects of ERP1.14a and ERP1.28a on subcellular localization of TDP-43 in FTLT-DTP lymphoblasts. Control and *GRN* mutation carrier lymphoblasts were

seeded at an initial density of 1×10^6 cells $\times$ ml⁻¹ and incubated in presence or absence of ERP1.14a and ERP-1.28a (10 μ M) for 24 hours. After treatment, lymphoblasts were collected and lysed to obtain the cytosolic (A) and nuclear (B) fragments that were analyzed by Western blotting. α -tubulin and LaminB1 antibodies were used as loading and purity control of the cytosolic and nuclear fractions respectively. A representative experiment is shown. Densitometric analyses represent the mean $\pm$ SEM of different observations carried out in cell lines (n=5) from each group (*p<0.05 and ***p<0.001 significantly different from control cells. ††p<0.01, significantly different from untreated cells)

Figure 6.- Effects of ERP1.14a and ERP1.28a on subcellular localization of TDP-43 in ALS lymphoblasts. Control and *ALS* lymphoblasts were seeded at an initial density of 1×10^6 cells $\times$ ml⁻¹ and incubated in presence or absence of ERP1.14a and ERP1.28a (10 μ M) for 24 hours. After treatment, lymphoblasts were collected and lysed to obtain the cytosolic (A) and nuclear (B) fragments that were analyzed by Western blotting. α -tubulin and LaminB1 antibodies were used as loading and purity control of the cytosolic and nuclear fractions respectively. A representative experiment is shown. Densitometric analyses represent the mean $\pm$ SEM of different observations carried out in cell lines (n=5) from each group (*p<0.05 significantly different from control cells. †p<0.05 significantly different from untreated cells)

Figure 7.- Effects of ERP1.14a and ERP1.28a on CDK6 expression levels, and proliferative activity in control, FTLT-TDP and ALS lymphoblasts. Immortalized lymphocytes from control and c.709-1G>A *GRN* mutation carriers, and ALS patients were seeded at an initial density of $1 \times 10^6 \times$ ml⁻¹ and cultured in RPMI medium

containing 10% FBS in the absence or in the presence of ERP1.14a and ERP1.28a (10 μ M). 24 hours later cells were harvested to isolate RNA and to prepare cell lysates. A) *CDK6* mRNA expression levels were analyzed by quantitative RT-PCR. Data shown are the mean $\pm$ SEM of different observations using cell lines derived from control individuals (n=5), FTLD-TDP patients (n=5), and ALS patients (n=5). B) Representative immunoblot showing CDK6 protein content after drugs treatment. Densitometric measurements were performed on individual immunoblots and values indicate the mean of CDK6 levels normalized with the corresponding β -actin levels $\pm$ SEM for experiments carried out with different cell lines (n=5) for each group. C) Cells were harvested after 72 of seeding for cell counting. **p<0.01 significantly different from control cells; †p<0.05, ††p<0.01 significantly different from untreated cells.

Figure 1

Figure 2

Figure 3

Figure 4

A **B**

Figure 5

Figure 6

A **B** **C**

Figure 7