NEURAL REGENERATION RESEARCH
www.nrronline.org

MAP4K inhibition as a potential therapy for amyotrophic lateral sclerosis

Shuaipeng Ma, Chun-Li Zhang*

Amyotrophic lateral sclerosis (ALS) is a rare neurological disease, featuring gradual loss of muscle controls due to degeneration of motor neurons. Unfortunately, there is currently no cure for ALS. The available therapies only offer a limited extension of survival by several months, begging for more options of therapeutics.

Since ALS is an adult-onset neurodegenerative disease, aging-relevant human motor neurons will be superior in modeling the disease and identifying potential therapeutics. In recent years, scientists have been able to directly convert human skin fibroblasts to generate patient-specific neurons through various strategies involving the manipulation of key fate-determining factors. One notable feature of these neurons is that they retain aging-associated characteristics that are reflected on transcriptomics, epigenetics, and mitochondria (Kim et al., 2018; Ma et al., 2020; Mertens et al., 2021). This contrasts with neurons derived from induced pluripotent stem cells, which are reset to an embryonic stage and lose aging-associated cellular features. The application of aging-relevant human neurons may help explore and identify the most promising mechanisms and target pathways in neurodegenerative diseases.

By employing aging-relevant human-induced motor neurons (hiMNs) from ALS patients (Liu et al., 2016), we recently conducted high-throughput screens of a chemical library of bioactive small molecules. Through this approach, we identified a neuroprotective compound and demonstrated that MAP kinase kinase kinase kinases (MAP4Ks) may serve as therapeutic targets for treating ALS (Liu et al., 2023). The lead compound, Hit3, formally known as K02288 (Sanvitale et al., 2013), functions as an inhibitor of MAP4Ks and regulates the MAP4Ks-HDAC6-TUBA4A-RANGAP1 pathway to restore the subcellular distribution of RAN GTPase-activating protein (RANGAP1) and the disease-associated protein TAR DNA-binding protein 43 (TDP-43). Ultimately, MAP4K inhibition preserves motor neurons and extends the lifespan of an animal model of ALS (**Figure 1**).

Trophic factor withdrawal, endoplasmic reticulum-stress-inducing compounds, and mutant SOD1-bearing astrocytes have been utilized as stimuli in neurons to simulate neurodegeneration (Yang et al., 2013; Larhammar et al., 2017; Bos et al., 2019; Wu et al., 2019). During our neuron purification process, we observed that ALS-hiMNs are differentially sensitive to systemic stress (Liu et al., 2016); within 3 days post-replating, over 50% of ALS-hiMNs succumbed to cell death. Based on this observation, we devised a survival assay. In our initial pilot screen using ALS-hiMNs, the glycogen synthase kinase-3 inhibitor kenpaullone (Yang et al., 2013) emerged as a chemical compound exhibiting neuroprotective effects (Liu et al., 2016). This proof-of-concept study suggests that ALS-hiMNs can be utilized for drug validation as well as chemical screens.

In our current screen of a library consisting of approximately 2000 compounds, including U.S. Food and Drug Administration-approved drugs and bioactive chemicals, we identified a lead compound and named Hit3. Hit3 demonstrated significant promotion of survival, growth, and function in aging-relevant ALS-hiMNs (Liu et al., 2023). These aging-relevant human neurons have not been previously employed for high-throughput chemical screens, primarily due to limited neuronal purity and yield. However, with improved conversion efficiency and simplified purification procedures, our recent work demonstrates the feasibility of utilizing these neurons for screening and investigating the underlying mechanisms (Liu et al., 2016, 2023). We conducted three sequential screens using ALS-hiMNs from a patient with FUS mutation. The vehicle (dimethyl sulfoxide) and Ken served as the negative and positive controls, respectively. Briefly, highly pure (> 85%) ALS-hiMNs were replated at about 1000 cells/well in Matrigel-coated 96-well plates 14 days post virus infection. The primary screens were finished three days post a single treatment of individual compounds at a final concentration of 2.5 μM. Viable cells were determined by CellTiter-Glo luminescent assays. The assay quality for each plate was evaluated by the Z-prime value. Screened chemicals were then ranked based on their effects on the relative survival of ALS-hiMNs. Based on the primary results, 65 top candidates (≥ 1.5 standard deviations above the mean of all tested compounds) were selected for secondary screens. Together with 23 additional inhibitors targeting TGF-β or glycogen synthase kinase-3, the candidate chemicals were screened at four concentrations: 0.5, 1.0, 2.5, and 5 μM. The top 15 chemicals (≥ 3 standard deviations above the mean of all tested compounds) were further examined in a tertiary screen for their dose responses (7 points in triplicates) in ALS-hiMNs. Their long-term effects were also examined in ALS-hiMNs cocultured with wild-type primary astrocytes for weeks. These screens revealed that Hit3 exhibited the most pronounced protective effects than any other chemicals. Such positive effects of Hit3 were also confirmed in ALS-hiMNs from patients with diverse mutations.

Identifying the direct kinase target of Hit3 could expedite the development of drugs for ALS. Hit3 was initially discovered as a potent inhibitor of the bone morphogenetic protein/activin-like kinase signaling pathway (Sanvitale et al., 2013). However, a systematic examination of other inhibitors targeting this pathway rather indicates that Hit3-mediated neuroprotection is not attributed to activin-like kinase inhibition. Interestingly, kinome-wide analyses showed that Hit3 also inhibits HGK (MAP4K4), MINK1 (MAP4K6), and TNIK (MAP4K7), all of which belong to the GCK-IV family of the STE20 group kinases (Sanvitale et al., 2013). MAP4Ki (PF6260933; Bos et al., 2019), a structural analog of Hit3 and a more

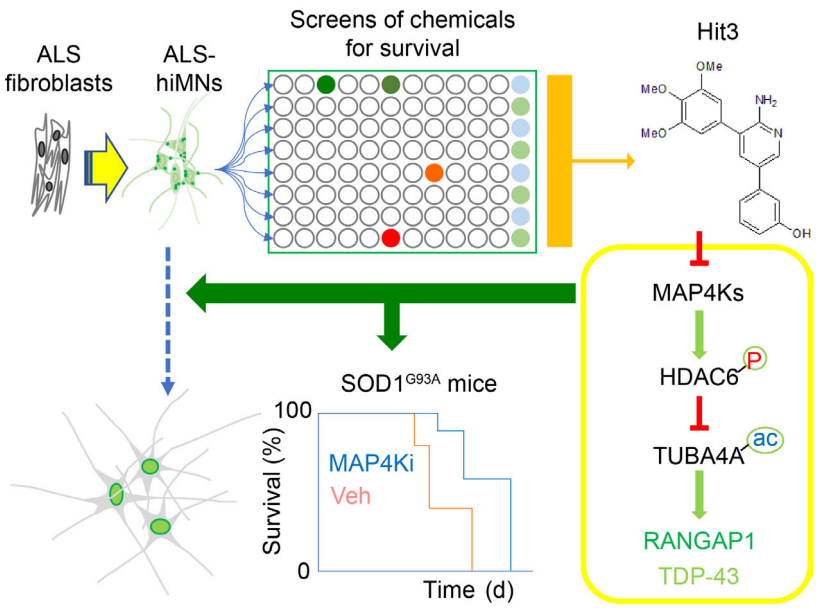

Figure 1 | **Identification and the action mechanism of a chemical for treating amyotrophic lateral sclerosis (ALS).** hiMNs were directly converted from patients' skin fibroblasts. ALS-hiMNs were used for large-scale screens of bioactive chemicals. The lead compound, Hit3, was identified as the most potent chemical for improving the survival and function of ALS-hiMNs. Mechanistically, Hit3 mainly functions as an inhibitor of MAP4Ks to block phosphorylation of HDAC6, resulting in enhanced acetylation of TUBA4A, stabilized microtubules, and nuclear localization of RANGAP1 and TDP-43. MAP4Ki could also significantly extend the lifespan of ALS mice. Created with Microsoft PowerPoint. HDAC6: Histone deacetylase 6; hiMNs: human-induced motor neurons; MAP4Ki: MAP4K inhibitor; MAP4Ks: MAP kinase kinase kinase kinases; RANGAP1: Ran GTPase-activating protein 1; SOD1: superoxide dismutase 1; TDP-43: TAR DNA-binding protein 43; TUBA4A: tubulin alpha-4A; Veh: vehicle.

selective MAP4K inhibitor, demonstrates a similar protective effect on ALS-hiMNs, suggesting that MAP4K inhibition may underlie Hit3's effect (Liu et al., 2023). Inhibition of these three kinases' expression or overexpression of their kinase-dead mutants validates the Hit3-mediated inhibition of MAP4Ks in improving the survival of ALS-hiMNs.

What are the downstream targets of MAP4Ks in ALS-hiMNs? Although MAP4Ks are known to regulate activation of p38 and JNK in induced pluripotent stem cell-derived human motor neurons or embryonic mouse dorsal root ganglion neurons (Yang et al., 2013; Larhammar et al., 2017; Bos et al., 2019; Wu et al., 2019), inhibitors specifically targeting these downstream kinases were found to be non-effective or toxic to the survival of ALS-hiMNs (Liu et al., 2023). Proximity-dependent biotin identification (BioID) is an established tool for identifying candidate targets within a bait protein's interactome. The fusion of BioID2 to MINK1 allows for the biotinylation of interacting or proximal proteins in the presence of biotin, which can subsequently be isolated and identified by mass spectrometry. By using BioID2-mediated protein proximity labeling and mass spectrometry, we conducted an unbiased identification of the MAP4K-associated proteins in ALS-hiMNs. This approach helped uncover that MAP4Ks may regulate multiple pathways, including Proteasome, Ribosome, and RNA Transport, which are known to be involved in neurodegenerative processes such as ALS.

In our study, we specifically focused on one potential target, RANGAP1, due to its abnormal subcellular distribution in ALS-hiMNs. We found that the introduction of Hit3 or dominant-negative MINK1 mutants significantly reduced RANGAP1-containing cytoplasmic foci and improved the nuclear localization of RANGAP1. RANGAP1 functions as the GTPase-activating protein of RAN and is involved in regulating nucleocytoplasmic transport (Raghunayakula et al., 2015). Interestingly, we observed that MAP4Ks do not directly regulate the phosphorylation of RANGAP1. This raises the question of how MAP4Ks control the transportation of the RANGAP1-associated protein complex between the cytoplasm and the nucleus.

It is known that RANGAP1 localizes to both nuclear pore complexes and annulate lamellae pore complexes, and its transportation requires the assistance of stable microtubules (Raghunayakula et al., 2015). Our data supports this, as we observed an exacerbated localization of RANGAP1 in cytoplasmic foci after suppressing MAP4K-associated tubulin alpha-4A (TUBA4A). This result is consistent with the report that mutations in TUBA4A can disrupt the microtubule network and are implicated in familial ALS (Smith et al., 2014). A hypothesis was then that MAP4Ks might destabilize TUBA4A through phosphorylation; however, this was not the case. Since the acetylation levels of alpha-tubulin correlate with increased microtubule stability and histone deacetylase 6 (HDAC6) is the major enzyme controlling tubulin acetylation, we then hypothesized that MAP4Ks might inhibit TUBA4A acetylation by enhancing the activity of HDAC6 through phosphorylation. This turned out to be the case, as our *in vitro* kinase assays showed that MAP4Ks can directly phosphorylate HDAC6. Phosphorylation of HDAC6 by MAP4Ks may increase its enzymatic activity or protein stability, as evidenced by our findings that inhibition of MAP4Ks or suppression of kinase expression promotes acetylation of TUBA4A. Furthermore, our data showed that the downregulation of HDAC6 promotes the nuclear localization of RANGAP1 and TDP-43 in ALS-hiMNs. Together, our findings suggest that MAP4Ks regulate the distribution of RANGAP1 via the HDAC6-TUBA4A axis (Liu et al., 2023).

Although previous studies also indicated that MAP4K inhibition could benefit the survival of *in vitro* cultured motor neurons (Yang et al., 2013; Bos et al., 2019; Wu et al., 2019), animal studies were not conducted. In our study, we investigated the *in vivo* role of MAP4K inhibition using the $SOD1^{G93A}$ mouse model of ALS (Liu et al., 2023). Because of its better blood-brain barrier penetration than Hit3, MAP4Ki was selected for animal studies. Intrathecal injections were selected as the administration route to further enhance drug accessibility to the nervous system. Excitingly, MAP4Ki-treated ALS mice showed a significantly extended lifespan compared to the vehicle control group. These mice also preserved a higher number of $CHAT^{+}$ motor neurons and more cells with normal expression patterns of RANGAP1 and TDP-43.

Overall, this is the first application of aging-relevant human neurons in large-scale chemical screens. Our screens identified a small molecule that remarkably improves the survival of human ALS patient-derived motor neurons. Our follow-up mechanistic studies further reveal the target kinases and the kinase-regulated signaling pathways. Importantly, *in vivo* kinase inhibition significantly extends the lifespan of ALS animals. Our work reveals the value of aging-relevant human neurons in therapeutics identification and mechanistic studies. Future medicinal chemistry is needed to develop the lead compound as therapeutics for human ALS patients. Mutant forms of the kinases could also be developed as a gene therapy due to their dominant negative effects and improving survival of human ALS motor neurons.

CLZ is a W.W. Caruth, Jr. Scholar in Biomedical Research. This work was supported by the TARCC, Welch Foundation Award (I-1724), the Decherd Foundation, the Pape Adams Foundation, and NIH grants NS092616, NS127375, NS117065, and NS111776 (to CLZ).

Shuaipeng Ma, Chun-Li Zhang*
Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA (Ma S, Zhang CL)
Hamon Center for Regenerative Science and Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA (Ma S, Zhang CL)
***Correspondence to:** Chun-Li Zhang, PhD, Chun-Li.Zhang@UTSouthwestern.edu.
https://orcid.org/0000-0002-2639-4605 (Chun-Li Zhang)

Date of submission: July 11, 2023
Date of decision: September 20, 2023
Date of acceptance: October 10, 2023
Date of web publication: December 11, 2023
https://doi.org/10.4103/1673-5374.389639
How to cite this article: *Ma S, Zhang CL (2024) MAP4K inhibition as a potential therapy for amyotrophic lateral sclerosis. Neural Regen Res 19(8):1639-1640.*

References

Bos PH, Lowry ER, Costa J, Thams S, Garcia-Diaz A, Zask A, Wichterle H, Stockwell BR (2019) Development of MAP4 kinase inhibitors as motor neuron-protecting agents. Cell Chem Biol 26:1703-1715.

Kim Y, Zheng XD, Ansari Z, Bunnell MC, Herdy JR, Traxler L, Lee H, Paquola ACM, Blithikioti C, Ku MC, Schlachetzki JCM, Winkler J, Edenhofer F, Glass CK, Paucar AA, Jaeger BN, Pham S, Boyer L, Campbell BC, Hunter T, et al. (2018) Mitochondrial aging defects emerge in directly reprogrammed human neurons due to their metabolic profile. Cell Rep 23:2550-2558.

Larhammar M, Huntwork-Rodriguez S, Rudhard Y, Sengupta-Ghosh A, Lewcock JW (2017) The Ste20 family kinases MAP4K4, MINK1, and TNIK converge to regulate stress-induced JNK signaling in neurons. J Neurosci 37:11074-11084.

Liu ML, Zang T, Zhang CL (2016) Direct lineage reprogramming reveals disease-specific phenotypes of motor neurons from human ALS patients. Cell Rep 14:115-128.

Liu ML, Ma S, Tai W, Zhong X, Ni H, Zou Y, Wang J, Zhang CL (2023) Chemical screens in aging-relevant human motor neurons identify MAP4Ks as therapeutic targets for amyotrophic lateral sclerosis. bioRxiv. doi: 10.1101/2023.04.24.538014.

Ma S, Zang T, Liu ML, Zhang CL (2020) Aging-relevant human basal forebrain cholinergic neurons as a cell model for Alzheimer's disease. Mol Neurodegener 15:61.

Mertens J, Herdy JR, Traxler L, Schafer ST, Schlachetzki JCM, Bohnke L, Reid DA, Lee H, Zangwill D, Fernandes DP, Agarwal RK, Lucciola R, Zhou-Yang L, Karbacher L, Edenhofer F, Stern S, Horvath S, Paquola ACM, Glass CK, Yuan SH, et al. (2021) Age-dependent instability of mature neuronal fate in induced neurons from Alzheimer's patients. Cell Stem Cell 28:1533-1548.

Raghunayakula S, Subramonian D, Dasso M, Kumar R, Zhang XD (2015) Molecular characterization and functional analysis of annulate lamellae pore complexes in nuclear transport in mammalian cells. PLoS One 10:e0144508.

Sanvitale CE, Kerr G, Chaikuad A, Ramel MC, Mohedas AH, Reichert S, Wang Y, Triffitt JT, Cuny GD, Yu PB, Hill CS, Bullock AN (2013) A new class of small molecule inhibitor of BMP signaling. PLoS One 8:e62721.

Smith BN, Ticozzi N, Fallini C, Gkazi AS, Topp S, Kenna KP, Scotter EL, Kost J, Keagle P, Miller JW, Calini D, Vance C, Danielson EW, Troakes C, Tiloca C, Al-Sarraj S, Lewis EA, King A, Colombrita C, Pensato V, et al. (2014) Exome-wide rare variant analysis identifies TUBA4A mutations associated with familial ALS. Neuron 84:324-331.

Wu C, Watts ME, Rubin LL (2019) MAP4K4 activation mediates motor neuron degeneration in amyotrophic lateral sclerosis. Cell Rep 26:1143-1156.

Yang YM, Gupta SK, Kim KJ, Powers BE, Cerqueira A, Wainger BJ, Ngo HD, Rosowski KA, Schein PA, Ackeifi CA, Arvanites AC, Davidow LS, Woolf CJ, Rubin LL (2013) A small molecule screen in stem-cell-derived motor neurons identifies a kinase inhibitor as a candidate therapeutic for ALS. Cell Stem Cell 12:713-726.

C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y