IFB-088 improves motor neuron survival in ALS models by reducing TDP-43 cytoplasmic mislocalization

InFlectis BioScience

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Introduction

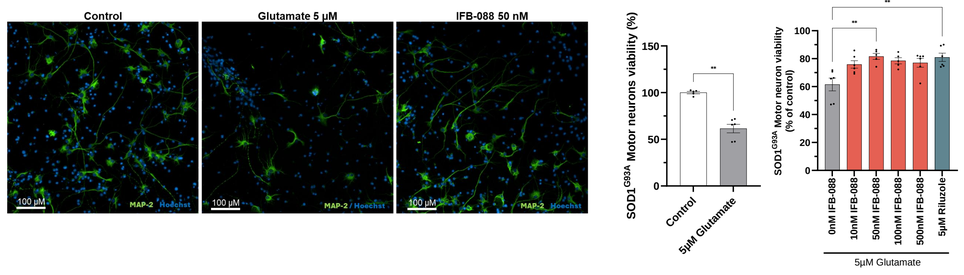
Amyotrophic lateral sclerosis (ALS) is characterized by abnormal cytoplasmic accumulation of misfolded proteins, such as TDP-43, and enlarged endoplasmic reticulum (ER), indicating ER stress. TDP-43 (TARDBP) forms pathological inclusions of phosphorylated and ubiquitinated protein in the cytoplasm of motor neurons of 97% of ALS patients. TDP-43, localized predominantly in the nucleus in normal physiological conditions, regulates transcription, pre-mRNA splicing and translation. In response to cellular stress, TDP-43 translocates into the cytoplasm where it can form cytoplasmic stress granules, which safeguard the essential mRNAs from degradation and promotes rapid recovery after stress removal. However, under prolonged stress, TDP-43 protein is found accumulated in the cytoplasm. Loss of nuclear TDP-43 function leads to abnormal RNA splicing and incorporation of erroneous cryptic exons which provoke the loss of crucial neuronal proteins such as Stathmin 2 or UNC13A. Increasing evidence suggests that the decrease in nuclear localization and the increase in cytoplasmic localization of TDP-43 induce toxicity through loss and gain of function mechanisms.

IFB-088 (icerguastat or Sephin1) is a first-in-class, brain penetrant, orally available small molecule, which has completed a phase 2 clinical trial in bulbar onset ALS patients (NCT05508074, TRIALS). IFB-088 has been described to be an inhibitor of stress-induced phosphatase complex. By inhibiting the stress-induced phosphatase complex and therefore prolonging eIF2α phosphorylation, IFB-088 prolongs the Integrated Stress Response pathway aiming to restore protein and cellular balance to prevent cell death. Despite the evidence that IFB-088 could improve proteostasis, motor neuron survival and motor function in ALS model (Das et al., 2015, Science) , no evidence proves that IFB-088 could reduce TDP-43 cytoplasmic localization or TDP-43 toxicity in ALS models.

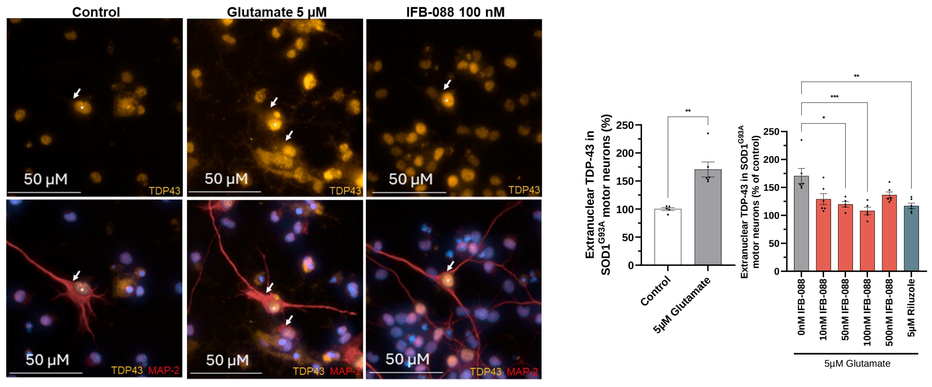
First, the impact of IFB-088 on TDP-43 toxicity, in particular its localization, was evaluated in motor neurons following glutamate excitotoxicity. Second, the impact of IFB-088 on abnormal splicing due to nuclear TDP-43 depletion function was evaluated in human cell line, SH-SY5Y following oxidative stress. Third, IFB-088 was evaluated in 2 *in vivo* ALS animal model. The impact of IFB-088 on motor neuron survival and TDP-43 aggregates was evaluated in the SOD1^{G93A} mouse model and in the TDP-43 transgenic zebrafish model. Finally, the level of plasmatic EVs TDP-43 was evaluated in patients who received IFB-088 for 6 months during the phase 2 clinical trial (NCT05508074).

IFB-088 improves motor neurons survival and reduces extra-nuclear TDP-43 localisation following glutamate intoxication

Motor neuron Survival



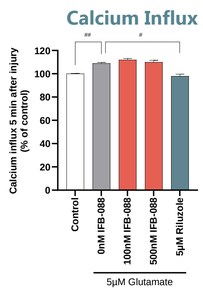
TDP-43 extra-nuclear localisation



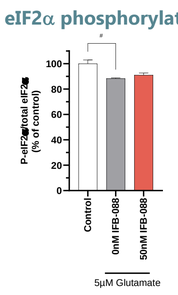
IFB-088, riluzole 5µM and DMSO were applied on primary SOD1^{G93A} rat motor neurons 1 hour before the addition of 5µM of glutamate. After 20 minutes of glutamate intoxication, glutamate was removed and replaced by fresh medium containing IFB-088, riluzole 5µM and DMSO for the next 24 hours. At the end of the incubation period, the cells are fixed and labelled with anti-MAP2 antibody, anti-TDP-43 antibody and Hoechst. Neuron survival was measured by counting the number of MAP-2 labelled cells per well. The extranuclear TDP-43 analysis was performed by measuring in µm^2 the overlapped area between MAP-2 and cytoplasmic TDP-43 staining without the overlapped area between Hoechst and TDP-43 staining. * p<0,05 Mann-Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparison test

IFB-088 reduces mitochondrial ROS without modulating eIF2α phosphorylation and calcium influx

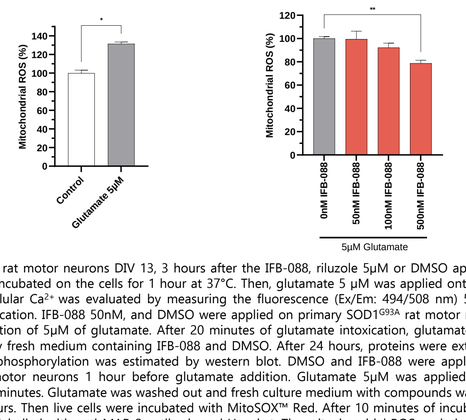
Calcium Influx



eIF2α phosphorylation

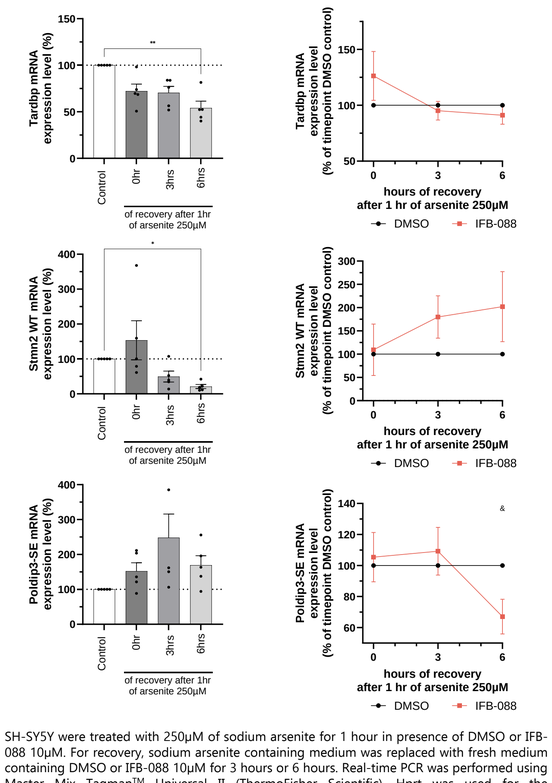


Mitochondrial ROS



On primary WT rat motor neurons DIV 13, 3 hours after the IFB-088, riluzole 5µM or DMSO application, 4 µM Fluo 4AM was incubated on the cells for 1 hour at 37°C. Then, glutamate 5 µM was applied onto the cells. The level of intracellular Ca^{2+} was evaluated by measuring the fluorescence (Ex/Em: 494/508 nm) 5 minutes after glutamate application. IFB-088 50nM, and DMSO were applied on primary SOD1^{G93A} rat motor neurons 1 hour before the addition of 5µM of glutamate. After 20 minutes of glutamate intoxication, glutamate was removed and replaced by fresh medium containing IFB-088 and DMSO. After 24 hours, proteins were extracted and the level of eIF2α phosphorylation was estimated by western blot. DMSO and IFB-088 were applied on primary SOD1^{G93A} rat motor neurons 1 hour before glutamate addition. Glutamate 5µM was applied on the motor neurons for 20 minutes. Glutamate was washed out and fresh culture medium with compounds was added for an additional 4 hours. Then live cells were incubated with MitoSOX™ Red. After 10 minutes of incubation, the cells were fixed and labelled with anti-MAP-2 antibody and Hoechst. The mitochondrial ROS analysis was performed by measuring in µm^2 the overlapped area between MAP-2 and MitoSOX staining. * p<0,05 Mann-Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparison test .

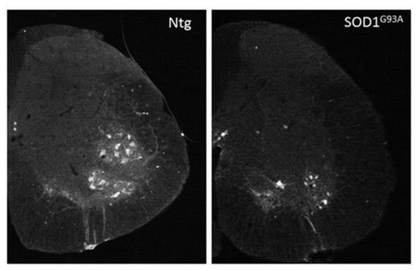
IFB-088 reduces abnormal splicing

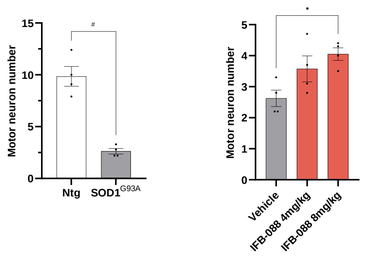


SH-SY5Y were treated with 250µM of sodium arsenite for 1 hour in presence of DMSO or IFB-088 10µM. For recovery, sodium arsenite containing medium was replaced with fresh medium containing DMSO or IFB-088 10µM for 3 hours or 6 hours. Real-time PCR was performed using Master Mix Taqman™ Universal II (ThermoFisher Scientific). Hprt was used for the normalization of mRNA level expression of Tardbp, Stmn2 WT and PoldIP3-SE. * p<0.05 Friedman test followed by Dunn's multiple comparison test, * p<0.05 é-Way ANOVA followed by Sidak's multiple comparison test

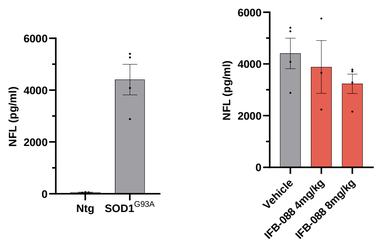
IFB-088 improves motor neurons survival and reduces TDP-43 in TIF in spinal cord of SOD1^{G93A} mice

Motor neuron count

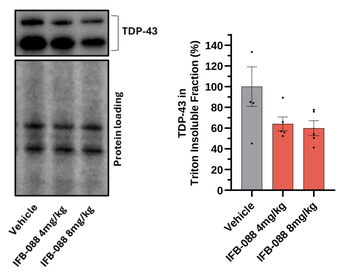




Plasmatic NFL level



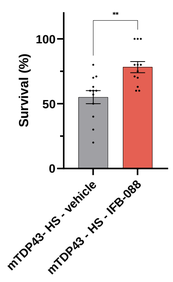
TDP-43 expression level in TIF



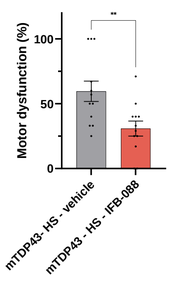
8 week-old female SOD1^{G93A} mice received by daily oral gavage IFB-088 4mg/kg, IFB-088 8mg/kg or vehicle (saline solution) for 12 weeks. After 12 weeks of treatment, mice were euthanized, plasma and spinal cord were collected for biochemical and histological analyses. Choline O-acetyltransferase (ChAT) immunostaining was performed on spinal cord sections (segments L2–L5, one every ten sections) using a primary anti-ChAT antibody following a standard immunofluorescence protocol. ChAT-immunopositive neurons with a cell body area >400 µm2 were counted in each hemisection. The NfL level in plasma were measured using the Simoa® NF-light™ Advantage (SR-X) Kit (#103400) on the Quanterix SR-X™ platform. Detergeant-Insoluble fraction (TIF) was extracted from the spinal cord. TDP-43 level in TIF Fraction was estimated by western blot. * p<0.05 Mann-Whitney test, * p<0.05 Kruskal-Wallis test followed by Dunn's multiple comparison test

IFB-088 improves motor neurons, reduces motor deficit and improves animal survival of mutant TDP-43 zebrafish embryos.

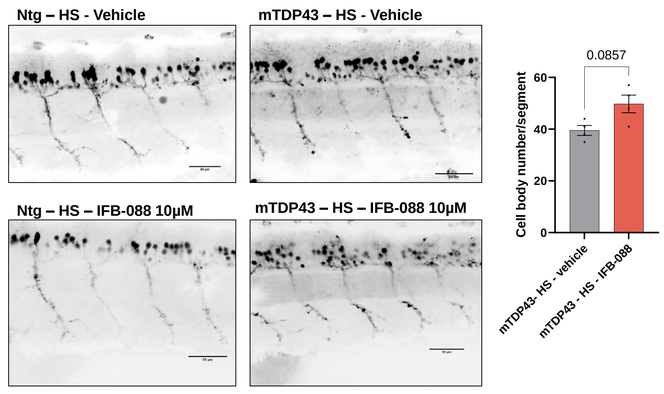
Survival



Motor dysfunction



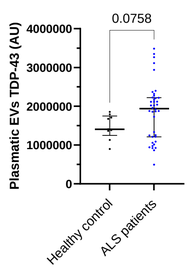
Motor neuron number

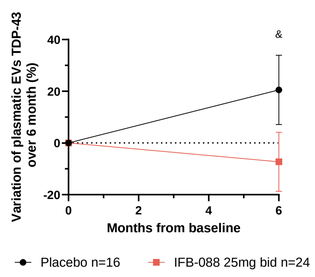


Mutant TDP-43 zebrafish embryos stably expresses mutant TDP-43^{G348C} protein following 2 heat shocks, the 1st one applied at 32 hpf and the 2nd one at 48 hpf. IFB-088 10µM or vehicle (DMSO) are applied to zebrafish embryos immediately after the first heat shock at 32 hpf. The treatment continued for 18-20 hours until motor function assessment and immunohistology analysis at 50-51hpf. Locomotor phenotypes of 50 hpf zebrafish embryos were assessed using the Touched-Evoked Escape Response (TEER) test. Mutant TDP-43 transgenic zebrafish were crossed with Tg(UAS:RFP) zebrafish to generate double transgenic zebrafish line having a specific expression of RFP in motor neurons allowing the observation of cell bodies of single motor neurons and their axonal arborization within a somatic segment in fixed and lived animals. * p<0.05 Mann-Whitney test

IFB-088 reduces TDP-43 in plasma Extracellular Vesicles from bulbar onset ALS patients

Demographics	Healthy control n=9	Placebo n=16	IFB-088 25mg *bid*- n=24
Sex, n(%)			
- Males	2 (22.22%)	11(68.75%)	13 (54.17%)
- Females	7 (77.78%)	5 (31.25%)	11 (45.83%)
Age (years)			
- Mean ± SD	24.78 (3.53)	62.38 ± 9.85	61.13 ± 8.97
- Median (range)	25 (20-32)	63.5 (45-75)	64 (35-74)
Time from 1st symptoms to screening (months)			
- Mean ± SD		11.31 ± 3.77	11.88 ± 4.34
- Median (range)		11 (6-17)	12 (5-18)
ALSFRS score at baseline			
- Mean ± SD		44 ± 2.10	42.54 ± 2.38
- Median (range)		45 (38-46)	43 (37-46)





Bulbar-onset ALS patients were treated daily with placebo plus riluzole 100mg or IFB-088 25mg bid plus riluzole 100mg for 6 months during the phase 2 clinical trial (NCT05508074). TDP-43 protein was detected by mass spectrometry in purified plasma Extracellular Vesicles (EVs) from 9 healthy controls and from 40 bulbar onset ALS patients, 16 placebo treated patients and 24 IFB-088-treated patients at baseline and after 6 months of treatment. * p<0.05 2-Way ANOVA followed by uncorrected Fisher's LSD

Conclusion

IFB-088 protects motor neurons against glutamate excitotoxicity through a mechanism likely dependent on the reduction of mitochondrial ROS. This neuroprotective effect of IFB-088 on motor neuron survival is also observed in the spinal cord of SOD1^{G93A} mice and in TDP-43^{G348C} expressing zebrafish embryos. In motor neurons, IFB-088 reduces the accumulation of TDP-43 proteins in the cytoplasm, thereby contributing to the neuroprotective action of IFB-088. It reduces abnormal splicing due to TDP-43 nuclear loss of function which could also contribute to IFB-088 neuroprotective action. In an animal model dependent on TDP-43 toxicity, IFB-088 improves motor function and prolongs the survival of mutated TDP-43 zebrafish model. In bulbar onset ALS patient, a 6-month treatment of IFB-088 25mg bid reduces the presence of TDP-43 in extracellular vesicles compared to placebo treated group.

IFB-088 improves motor neuron survival in multiple preclinical ALS models by reducing TDP-43 cytoplasmic mislocalization and reducing abnormal splicing due to TDP-43 nuclear loss of function. This study demonstrates, for the first time, that IFB-088 was able to decrease TDP-43 cytoplasmic mislocalization not only in preclinical ALS model but also in plasma EVs from ALS patients.