Oral treatment with MEK-inhibitors decreases Nfix in adult dystrophic muscles: a pilot study for drug-repurposing in Muscular Dystrophy

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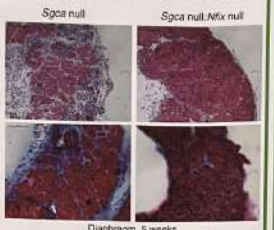
A PHARMACOLOGICAL APPROACH TO TREAT MUSCULAR DYSTROPHIES?

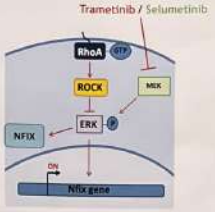
Muscular Dystrophies (MDs) are incurable monogenic myopathies characterized by progressive degeneration of skeletal muscle. Dystrophic mice lacking the transcription factor **Nfix**, crucial for switching from embryonic to fetal myogenesis, display both morphological and functional improvements of the disease, due to the slowing down of muscle regeneration and to a shift towards more oxidative myofibers (Rossi et al. 2017).

Recently, we demonstrated that the **MAPK (MEK/ERK)** signaling pathway positively regulates Nfix in fetal myoblasts *in vitro* and *in vivo*, bringing out the idea of an indirect pharmacological inhibition of Nfix in the MD context (Taglietti et al. 2018).

To this purpose, we selected two different **MEK-inhibitors**, Trametinib and Selumetinib, already used in clinic as anti-cancer drugs. Moreover, Selumetinib was tested in a murine model of Emery-Dreifuss Muscular Dystrophy (EDMD) with promising results.

This research project aimed to verify whether the inhibition of the MEK/ERK pathway by Trametinib/Selumetinib decrease the expression of Nfix also in dystrophic muscles leading to histological improvements of the disease.





MEK-INHIBITOR DECREASES THE NFIX PROTEIN LEVEL IN POSTNATAL MYOBLASTS *IN VITRO*

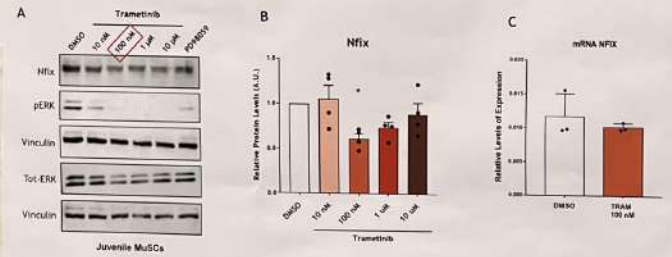
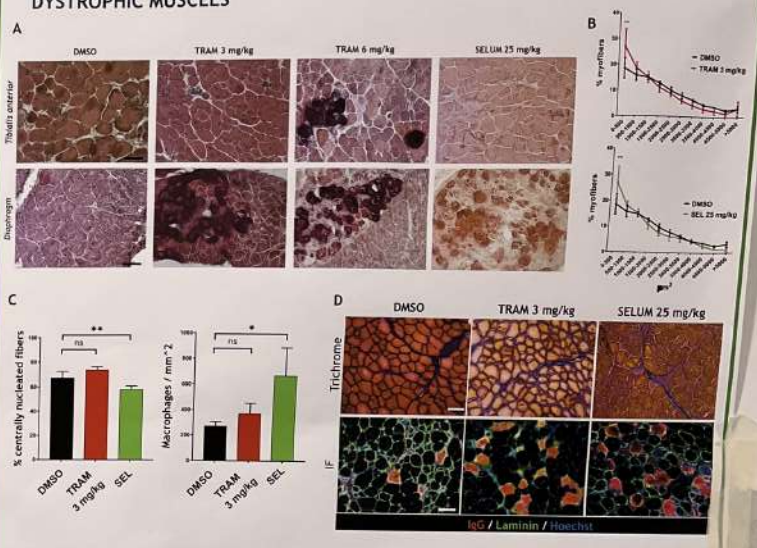


Figure 1. ***In vitro*** **treatment with Trametinib leads to a decrease in pERK and Nfix protein in juvenile MuSC-derived myoblasts.** (A) Representative western blots of juvenile myoblasts treated with DMSO and different concentration of Trametinib *in vitro*, revealing protein levels of Nfix, ERK (pERK, tot-ERK), and Vinculin used as housekeeping protein. PD98059-treated myoblasts were used as positive control. (B) Quantitative densitometry of Nfix protein levels in juvenile myoblasts treated with DMSO or Trametinib at different concentrations (* P<0.05, paired one-way ANOVA test, n=4). (C) qRT-PCR for the expression of *Nfix* in juvenile MuSC-derived myoblasts treated with vehicle (DMSO) or 100 nM Trametinib for 14 h. *B-actin* was used as housekeeping gene (paired t-test with Welch's correction, n=3).

HISTOLOGICAL ANALYSIS OF TRAMETINIB- AND SELUMETINIB-TREATED DYSTROPHIC MUSCLES



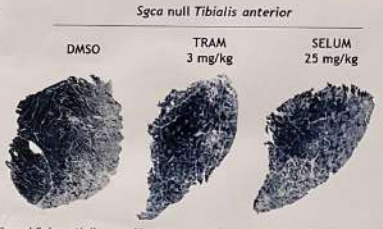


Figure 3. Effect of Trametinib and Selumetinib on caliber, regeneration, necrosis, inflammation, and metabolism of dystrophic myofibers. (A) Hematoxylin and eosin (H&E) staining of *Tibialis anterior* (top) and Diaphragm (down) muscles treated with DMSO, 3 and 6 mg/kg of Trametinib, and 25 mg/kg of Selumetinib every day for 14 days by oral gavage. Scale bar 100 µm for Tibialis anterior, 200 µm for Diaphragm (n=4 for each condition). (B) Quantifications of cross-sectional area distribution, (C *left*) centrally nucleated myofibers, and (C *right*) macrophages infiltration (F4/80+ cells) of *Sgca* null *Tibialis anterior* treated with DMSO (black), 3 mg/kg Trametinib (red), and 25 mg/kg Selumetinib (green) (** P<0,001, * P<0,01 one-way ANOVA test; n=4 for each condition). (D) Milligan's trichrome staining and immunofluorescence against murine IgG (red), Laminin (green) and nuclei (blue) of *Tibialis anterior* muscles chronically treated with DMSO, 3 mg/kg Trametinib, and 25 mg/kg Selumetinib every day for 14 days by oral gavage (Scale bar 200 µm; n=4 for each condition). (E) Succinate Dehydrogenase (SDH) staining on the entire *Sgca* null *Tibialis anterior* muscle sections upon chronic treatment with Trametinib, Selumetinib, and DMSO.

CHRONIC TREATMENT WITH TRAMETINIB CAUSES A REDUCTION OF pERK AND NFIX IN DYSTROPHIC MUSCLE

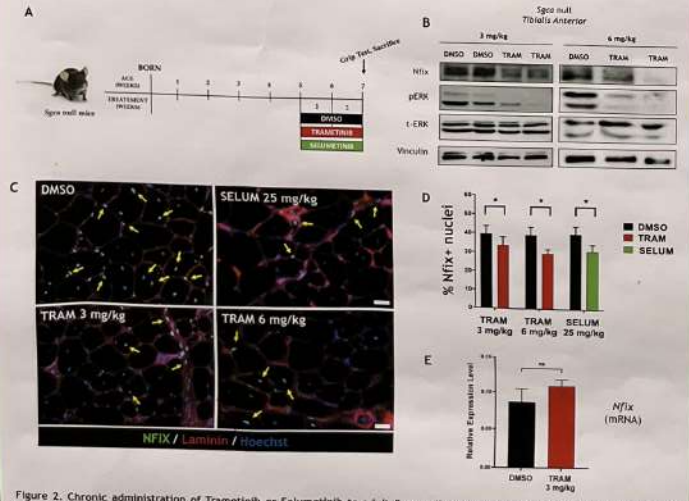


Figure 2. Chronic administration of Trametinib or Selumetinib to adult *Sgca* null mice by oral gavage induces a reduction of Nfix+ cells in dystrophic muscles. (A) Visual scheme of the chronic treatment protocol used to treat dystrophic animals. 3-6 mg/kg of Trametinib and 25 mg/kg of Selumetinib were administered to adult *Sgca* null mice (5 weeks old) by oral gavage, every day for 14 days. (B) Representative Western Blots of protein extracts from *Tibialis anterior* muscles of *Sgca* null mice treated with 3 and 6 mg/kg of Trametinib every day for 14 days, by oral gavage. (C) Representative immunofluorescence images of *Tibialis anterior* muscles isolated from adult *Sgca* null mice treated with DMSO, 3 and 6 mg/kg Trametinib, and 25 mg/kg Selumetinib (Nfix = green, Laminin = red, Hoechst = blue). (D) Graph depicting the percentage of Nfix+ nuclei on the total number of nuclei per section in 3, 6 mg/kg Trametinib, and 25 mg/kg Selumetinib than DMSO (vehicle)-treated muscles (* P<0.05, Unpaired t-test with Welch's correction, n=4). (E) qRT-PCR for expression of *Nfix* in *Tibialis anterior* treated with vehicle (DMSO) or 3 mg/kg Trametinib every day for 14 days by oral gavage. *B-actin* was used as housekeeping gene (Unpaired t-test with Welch's correction, n=3).

TRAMETINIB INDUCES MUSCULAR CALCIFICATIONS IN DYSTROPHIC BUT NOT IN WT MUSCLES

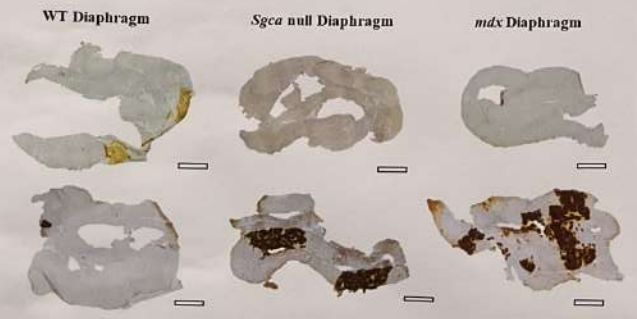


Figure 3. Chronic treatment with Trametinib induces muscular calcifications in dystrophic muscles regardless of the genetic background. (A) Representative total-muscle Alizarin Red staining of Diaphragms isolated from WT, *Sgca* null and *mdx* treated with 3 mg/kg Trametinib every day for 14 days by oral gavage. Muscular calcifications are evidenced as red areas inside the tissue.

CONCLUSIONS

- ✓ Nfix modulation by MEK/ERK pathway occurs at post-translational level in myogenic cells and in dystrophic muscles;
- ✓ Chronic administration of Trametinib or Selumetinib, every day for 14 days by oral gavage, reduces the Nfix protein levels in adult dystrophic muscles
- ✓ Slight increase of oxidative phenotype of dystrophic myofibers;
- ✓ Nfix reduction is not sufficient for histological ameliorations;
- ✓ Trametinib and Selumetinib causes calcified myofibers (high dosages and in Diaphragm, particularly)

FUTURE PERSPECTIVES

- ➢ Two weeks of drug administration might be not sufficient: longer period of drug administration;
- ➢ Too compromised muscle tissue in adult *Sgca* null mice: early MEK-inhibition in young dystrophic mice (3 week-old);
- ➢ Further analyses on parallel MAPK pathways: like JNK and p38 signaling pathways;
- ➢ Combination between MEK inhibitors and the cyanidin-reach diet

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