A Potential Novel Treatment for Neurofibromatosis Type 1 via RAS Inhibition

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Introduction

Neurofibromatosis type 1 is a genetic disease that results from either heritable or spontaneous autosomal dominant mutations in the NF1 gene. Neurofibromatosis type 1 individuals frequently suffer benign tumors known as Plexiform Neurofibromas which develop from cranial and peripheral nerve sheaths. Plexiform Neurofibromas have the potential to develop into a highly deadly malignant peripheral nerve sheath tumor (MPNST). As low as thirty-four percent of individuals survive MPNST for more than 5 years and there is no effective treatment or cure.

The NF1 gene encodes the protein Neurofibromin. Neurofibromin is a negative regulator (a GAP) for the notorious RAS oncoprotein. Thus, inactivation of NF1 leads to a constitutive increase in the active form of RAS. This is a transforming event that drives the disease.

In an attempt to combat the problem of a lack of a therapeutic treatment for Neurofibromatosis Type 1, and indeed, RAS driven cancer in general, the Clark and Trent laboratories have performed *in silico* screening of two million compounds followed by bioassay to identify a small molecule, referred to as F3, that binds and inhibits active RAS. The compound is effective against models of mutant RAS driven tumor formation *in vitro* and *in vivo*.

Here, for the first time, we test the compound against a disease driven by hyper-activation of the wild type RAS protein, rather than the mutant form. Therefore, in this project we hypothesize that the use of F3 will limit RAS activated signaling of its mitogenic pathways in Neurofibromatosis Type 1 causing a suppression of cell growth and tumorigenicity. We aim to determine a mechanism for which this occurs as well as evaluate the functionality of F3 and its derivative in both two-dimensional and more physiologically relevant three-dimensional tissue culture assays of Neurofibromatosis Type 1 cell systems.

Methods

Cell Lines: IPN and S462.TY cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin in an incubator at 37˚ C in 5% CO2.

Proliferation Assays: IPN cells were seeded in the wells of a 12 well plate in a paired set where one 12 well plate received 5μM F3 treatment and the other plate received a DMSO control. S462.TY cells were seeded in the same manner in addition to a third 12 well plate in which 10μM F3 treatment was administered. Cell counts were taken every 24 hours using a hemocytometer.

Soft Agar Colony Formation Assays: S462.TY cells were treated with 10, 5, 1, and 0.1 μM concentrations of F3 in duplicate wells of a 12 well plate as well as a DMSO control. Cells were then suspended in a 0.6% agar solution by seeding on a 0.3% agar base. The cells were incubated at 37˚ C for two weeks before subsequent visualization using photography and quantification of present colonies.

Western Blotting: Protein samples were ran on 4-12% Bis-Tris gels and transferred on to 0.2μM nitrocellulose membranes. After antibody incubation, protein was visualized using SuperSignal West Pico Chemiiluminescent substrate.

Results

Neurofibromatosis is caused by mutations in NF1 leading to aberrant RAS activation

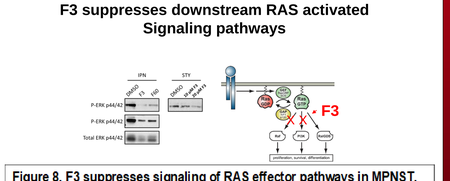

Figure 1. NF1 gene function in RAS activity. The NF1 gene encodes neurofibromin which works as a negative regulator to switch activated RAS-GTP to inactive RAS-GDP. If this function is lost due to mutation then RAS activity dominates.

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Figure 2. Pathogenesis of Neurofibromatosis Type 1. Due to a loss in function mutation to the NF1 gene. Normal Schwann cells exhibit proliferation into benign plexiform neurofibromas which is a precursor to malignant peripheral nerve sheath tumors.

Identification of a novel small molecule designated F3 that binds RAS

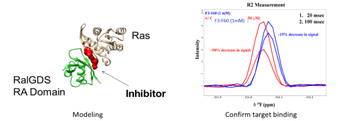

Figure 3. Blocking the interaction of RAS with its effectors. Through *in silico* screening of 2 million compounds, the Clark and Trent lab identified compound F3(left panel) as an inhibitor of RAS binding to its domain. Medicinal chemistry led the Clark and Trent lab to subsequent finding of an enhanced derivative of F3, F3-F60 which exhibits enhanced RAS binding affinity as measured by NMR.

F3 has little effect on the growth of immortalized normal Schwann cells

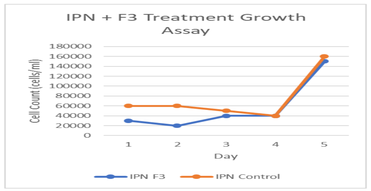

Figure 4. F3 Treatment of IPN cells. Schwann cells (IPN) treated with F3 showed little difference in growth when compared to IPN cells treated with DMSO control.

F3 slightly decreases growth of malignant peripheral nerve sheath tumor cells in 2-D

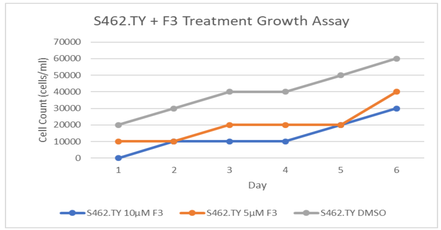

Figure 5. F3 Treatment suppresses S462.TY cell proliferation. Malignant peripheral nerve sheath tumor cells (S462.TY) treated with F3 exhibited slower growth progression at increased concentrations of F3 treatment.

F3 inhibits tumorigenicity in 3-D growth

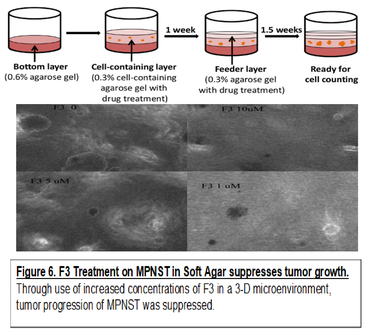

Figure 6. F3 Treatment on MPNST in Soft Agar suppresses tumor growth. Through use of increased concentrations of F3 in a 3-D microenvironment, tumor progression of MPNST was suppressed.

F3 exhibits more potency at increased concentrations

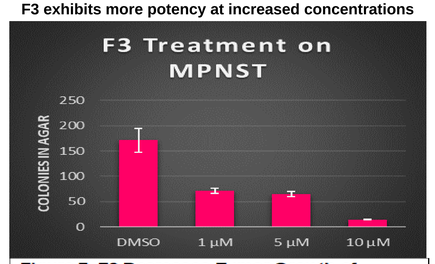

Figure 7. F3 Decreases Tumor Growth of MPNST in Soft Agar. Growth and proliferation of colonies in soft agar of malignant peripheral nerve sheath tumors was suppressed at higher concentrations of F3 dosage.

F3 suppresses downstream RAS activated Signaling pathways

Figure 8. F3 suppresses signaling of RAS effector pathways in MPNST. At increased concentrations of F3, a decrease was exhibited in the activity of the RAS-RAF-MEK-ERK signaling pathway.

Summary

1. Neurofibromatosis is caused by upregulation of RAS activity due to mutations in the negative Ras regulator NF1.
2. It leads to benign Plexiform Neurofibromas and malignant MPNST tumors.
3. There is currently no treatment or cure.
4. We have identified a novel small molecule that directly binds to active RAS and suppresses its function.
5. We now show the molecule has specific inhibitory activity Against MPNST tumors growing in 3D culture.
6. We propose the molecule may be developed into the first Therapy for NF1 disease.

Conclusions

In conclusion, the data suggests that use of the small molecule inhibitor F3 has little toxicity in 2-D growth but serves as a potent inhibitor of tumorigenic 3-D growth. Further analysis of the downstream signaling pathways associated with RAS activity showed suppression of RAS signaling. The findings highlight a possible therapeutic treatment for Neurofibromatosis Type 1 in which currently the only known form of treatment is surgical resection of the malignant tumors. In the future, the investigation of F3 and enhanced derivatives under development as a potential treatment for Neurofibromatosis Type 1 can be furthered by *in vivo* models ultimately leading to clinical trials.

Acknowledgements

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