Novel RAS inhibitors for NF1 disease

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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). MPNST exhibit a low overall 5 year survival rate of less then 40%, 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, we 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*. We have subsequently developed enhanced derivatives (F3F60).

Here, for the first time, we test the compounds against a disease driven by hyper-activation of the wild type RAS protein, rather than the mutant form. We find the compound has little effect on the growth of MPNST cells cultured in 2D but exhibits a striking inhibition of their ability to proliferate in 3D culture. We observe specific inhibition of RAS driven signaling pathways and suppression of tumor formation in vivo models of RAS mediated transformation, with no detectable toxicity. We propose this approach may lead to novel therapeutics for NF1 disease.

Results

Development of a RAS inhibitor for NF1 disease

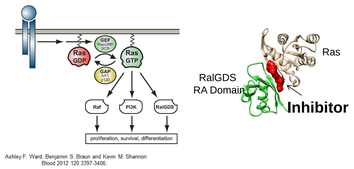

Figure 1. Development of an inhibitory strategy for NF1 disease.
LEFT: Ras activity is controlled by positive and negative regulators. NF1 is negative regulator, so inactivation of NF1 by mutation removes a "brake" on RAS resulting in an elevation of RAS activity.
RIGHT: using the known crystal structure of RAS in complex with its effector RALGDS, we used *in silico* screening to predict compounds which might bind to RAS and block its effector interactions.

Direct compound binding to RAS

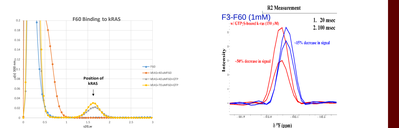

Figure 2. Compound F3 and derivatives directly bind to K-RAS.
Activated K-RAS protein was prepared and and used in direct compound binding assays. LEFT: Analytical Ultracentrifugation (AUC) was used to confirm co-sedimentation of F3 with GTP-loaded mutant K-RAS protein. RIGHT: Nuclear Magnetic Resonance (NMR) studies were performed to confirm compound binding. The binding profile demonstrated enhanced interaction when K-RAS was in the GTP bound (active) form.

F3 based compounds block the RAS/RAS interaction and specifically suppress RAS signaling.

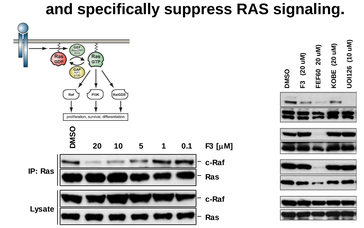

Figure 3. Inhibition of RAS effector interactions and signaling by compound F3 and F3 derivatives.
LEFT: Endogenous, mutant K-RAS was immunoprecipitated (IP) from MiaPaCa-2 cells in the presence or absence of various concentrations of F3. The complexes were immunoblotted (IB) for RAS and c-RAF to determine the effects on RAS/RAF binding. RIGHT: Panc1 cells were treated with drug for 1 hour before lysing and assaying the effects of F3 or enhanced derivative F3-F60 on the RAF/MAPK and PI3K/AKT pathways using phospho-antibodies.

Ras inhibitors have little effect on 2D cell growth

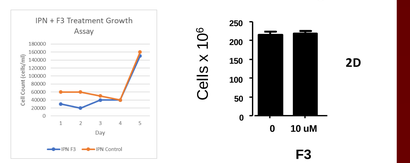

Figure 4. Treatment of immortalized Schwann cells or MPNST cells growing 1n normal 2D culture has little effect.
LEFT: Immortalized Schwann cells were cultured in drug or carrier and examined for cell growth characteristics.
RIGHT: S462.TY MPNST cells were incubated in drug or carrier for 1 week and then assayed to determine if there was any inhibition of growth.

F3 induces Anoikis in MPNST cells when cultured in 3D

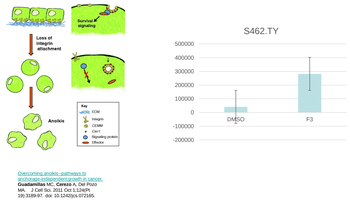

Figure 5. F3 induces Anoikis in MPNST cells when cultured in 3D.
LEFT PANEL: Anoikis is a process of apoptosis induced by cell suspension and the loss on integrin based survival signaling. Activation of RAS induces resistance to Anoikis, allowing tumors to form in 3D in the body.
RIGHT PANEL: Apoptosis is detected by caspase Glo assay in F3 treated MPNST cells cultured in 3D.

F3 inhibits MPNST soft agar growth

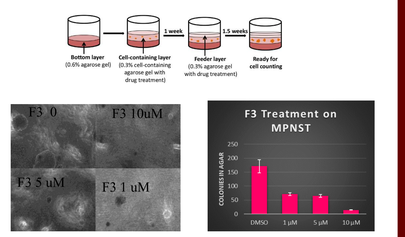

Figure 5. F3 potentially suppresses 3D growth of MPNST cells.
MPNST cells (S462.TY) were plated in soft agar in the presence or absence of F3 drug. Colony formation was scored after 2 weeks. An IC50 of ~ 1uM could be obtained.

F3 inhibits tumor growth without toxicity

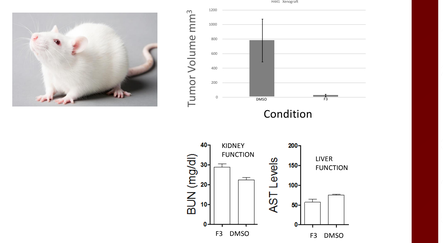

Figure 5. F3 suppresses tumor formation without toxicity in xenografts.
TOP: Muatnt Ras containing lung tumor cells (NCI-H441) were injected into the flank of NRG mice. After 48 hours, animals were ip injected with 20mg/kg of F3 every other day for four weeks. Tumor formation was assayed by measuring tumor volume. F3 treatment blocked tumor formation. BOTTOM: Kidney and Liver function were assayed in treated mice via BUN and AST levels. No toxicity was detected.

CONCLUSIONS

1. **We have identified a series of Novel RAS inhibitors.**
2. **The inhibitors have little toxicity in 2D but are effective in suppressing the tumorigenic phenotype of MPNST cells grown in 3D systems.**
3. **They are active in vivo against RAS driven tumors.**
4. **They exhibit no apparent in vivo toxicity.**
5. **These agents may be developed into novel targeted therapy for NF1 disease.**

Discussion

NF1 disease is largely caused by deregulation of RAS due to loss of function of NF1. We have developed a RAS binding small molecule (kd ~70uM) that inhibits the ability of RAS to interact with downstream effectors. Designated F3, the ~ 490kd compound has little effect at low concentrations on 2D cell growth but is a potent inhibitor of 3D growth of MPNST cells. This is likely due to the suppression of RAS signaling pathways leading to anoikis. The compound is effective at suppressing tumor formation by RAS driven lung tumor cell lines without inducing detectable *in vivo* toxicity. We have developed several enhanced activity derivatives by Medicinal Chemistry, which we are in the process of characterizing and optimizing further. We intend to test the compounds against *in vivo* models of MPNST, as well as *in vitro* models of PFN to determine if they have the potential to be developed into targeted therapy for NF1 disease. As at least some of the cognitive issues associated with NF1 patients also appear to be due to aberrant RAS activity, and as our compounds can pass the blood brain barrier, we also hypothesize they may have utility in treating neurological defects caused by excess RAS activity.

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