Award Number: W81XWH-12-1-0157

TITLE: Drug Response and Resistance in Advanced NF1-Associated Cancers

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REPORT DATE: May 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
May 2014	Annual	01 April 2013 – 31 March 2014

4. TITLE AND SUBTITLE
Drug Response and Resistance in Advanced NF1-Associated Cancers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0157

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, San Francisco
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Juvenile myelomonocytic leukemia (JMML) and other types of myeloproliferative neoplasms (MPNs) progress to acute myeloid leukemia (AML) in a substantial proportion of patients. The *NF1* gene is frequently inactivated in NF1 patients who develop either JMML or AML. However, AML is a more aggressive malignancy that invariably contains multiple additional genetic alterations that interact with *NF1* loss. We have extensively characterized MPN and AML in *Nf1* mutant mice and have investigated mechanisms of drug responses and resistance. Our studies of MEK inhibitors in *Nf1* mutant mice with MPN and AML showed that cooperating mutations that are acquired as MPN evolves to aggressive AML increase the dependence of these cells on Raf/MEK/ERK signaling. However, drug resistant AML clones rapidly emerge *in vivo*. We have also shown that we can utilize this novel system to validate genes that cause resistance to MEK inhibitors. Our goal is to deploy genetically accurate mouse models of advanced NF1-associated cancers to identify mutations that cooperate with *Nf1* inactivation in cancer progression, to develop a preclinical paradigm for combining conventional and targeted anti-cancer agents *in vivo*, and to uncover mechanisms of drug response and resistance. To date, we have generated a robust system for modulating gene expression in primary leukemia cells *in vivo*, generated data that support the hypothesis that AML cells remain dependent on Nf1 inactivation for robust growth, found that treatment with CPX-351 prolongs the survival of *Nf1* mice, and have begun testing combination regimens.

15. SUBJECT TERMS
neurofibromatosis, pediatric cancer, leukemia, targeted therapeutics, mouse models

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	20	

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INTRODUCTION

Malignant peripheral nerve sheath tumor (MPNST), high-grade astrocytoma, and acute myeloid leukemia (AML) are aggressive cancers cause premature mortality in patients with NF1. Despite recent advances in understanding the molecular genetics and underlying biology, current therapies for these malignancies remain ineffective.

Juvenile myelomonocytic leukemia (JMML) and other myeloproliferative neoplasms (MPNs) progress to acute myeloid leukemia (AML) in a substantial proportion of patients. The *NF1* gene is frequently inactivated in cases of JMML or AML that arise children with NF1. AMLs contain additional genetic alterations that interact with *NF1* loss. We have extensively characterized MPN and AML in *Nf1* mutant mice and have investigated mechanisms of drug response and resistance. Our studies of MEK inhibitors in *Nf1* mutant mice with MPN and AML showed that cooperating mutations that are acquired as MPN progress to AML unexpectedly increased the dependence of these cells on Raf/MEK/ERK signaling. However, drug resistant AML clones rapidly emerged *in vivo.* We have also shown that we can utilize this novel experimental system to validate genes that cause resistance to MEK inhibitors. By contrast, hematopoietic cells from *Nf1* mutant mice with MPN are less dependent on MEK for survival. Interestingly, while treatment with MEK inhibitors does not eliminate *Nf1* mutant cells in mice with MPN, it nonetheless induces remarkable hematologic improvement. Indeed, controlled preclinical trials in *Nf1* mutant mice have stimulated clinical evaluation of MEK inhibitors as single agents in JMML and in histologically benign human NF1-associated tumors such as plexiform neurofibroma. On the other hand, our studies of *Nf1* mutant mice with AML and data from patients with advanced cancers treated with tyrosine kinase inhibitors showed that drug resistance emerges rapidly. These data, in turn, support administering different targeted inhibitors together to simultaneously inhibit multiple signaling pathways and/or combining targeted and conventional cytotoxic drugs to treat advanced cancers in NF1 patients.

Our goal is to deploy a genetically accurate mouse models of NF1-associated AML to develop a preclinical paradigm for combining conventional and targeted anti-cancer agents *in vivo*, and to uncover mechanisms of drug response and resistance. We are pursuing three specific aims to achieve these objectives. These are:

(1) To restore neurofibromin GAP activity in primary murine AMLs to ask if this inhibits the growth of advanced cancers that are initiated by *Nf1* inactivation. We hypothesize that many cancers will remain dependent on hyperactive Ras signaling, but that some will evolve mechanisms that by-pass the requirement for neurofibromin expression.

(2) To develop treatment regimens employing both front-line chemotherapy and MEK inhibitors to treat a heterogeneous collection of primary murine AMLs that were initiated by inactivating the *Nf1* gene. We hypothesize that this will uncover synergistic inhibitory effects and will provide a rationale for testing this general approach in human patients with advanced NF1-associated cancers.

(3) To identify and validate genes and pathways underlying anti-cancer drug sensitivity and resistance in *Nf1* mutant AML. We hypothesize that these experiments will uncover novel mechanisms of drug resistance that will inform the design of clinical trials.

BODY

Background and Preliminary Studies

Tumorigenesis in Neurofibromatosis Type 1 (NF1). NF1 is a multi-system dominant genetic disorder caused by germ line mutations in the *NF1* tumor suppressor gene. *NF1* encodes a GTPase activating protein called neurofibromin that negatively regulates Ras signaling by accelerating the hydrolysis of active Ras-GTP to inactive Ras-GDP (1, 2). Clinical manifestations of NF1 include pigmented skin lesions, skeletal dysplasia, learning disabilities, and a propensity to develop benign and malignant tumors. The malignancies seen in NF1 patients include astrocytoma, malignant peripheral nerve sheath tumor (MPNST), pheochromocytoma, and childhood myeloid leukemia. A common feature of NF1-associated tumors is somatic loss of the normal *NF1* allele, which is consistent with its role as a tumor suppressor gene and with the biochemical function of neurofibromin as a negative regulator of Ras signaling (1, 2). Patients with NF1 who are cured of a primary cancer are at increased risk of developing treatment-induced secondary malignancies (3-6), and heterozygous *Nf1* mutant mice are predisposed to a spectrum of radiation-induced cancers (7, 8). Together, the benign neoplasms and more aggressive malignancies that develop in NF1 patients are a substantial cause of morbidity and premature mortality. There are currently no effective, mechanism-based therapies for any of the tumors that arise in persons with NF1.

Myeloid Malignancies in NF1. Children with NF1 are at greatly increased risk of developing juvenile myelomonocytic leukemia (JMML), an aggressive myeloproliferative neoplasm (MPN) characterized by over-production of differentiated myeloid lineage cells that show extensive tissue infiltration (9, 10). The median survival of JMML patients is <1 year without hematopoietic stem cell transplantation (HSCT), and the overall cure rate is ~50% after HSCT (11). Children with NF1 who develop JMML show distinct clinical features including older age at diagnosis and worse outcome (11). Our studies of JMML proved that *NF1* functions as a tumor suppressor gene in hematopoietic cells (12, 13), and provided the first direct evidence of deregulated Ras signaling in primary cancer cells from NF1 patients (14). The association of NF1 with JMML also implicated hyperactive Ras in the pathogenesis of this MPN, and our group and other investigators went on to discover germ line and somatic mutations in multiple components of Ras signaling networks in JMML patients (15-19). Despite the routine use of HSCT in JMML, up to 30% of patients progress to acute myeloid leukemia (AML). Evolution to AML may be associated with new cytogenetic changes such as monosomy 7 (20). In addition, myeloid malignancies are among the most common treatment-induced cancers diagnosed in children and adults with NF1 (3-5). Somatic *NF1* mutations are also increasingly recognized in patients with AML who do not have neurofibromatosis (21).

Modeling NF1-Associated Myeloid Malignancies in the Mouse. We collaborated with Dr. Luis Parada to generate *Mx1-Cre, Nf1*$^{flox/flox}$ mice, and injected them with polyinosinic-polycytidilic acid (pI-pC) to inactivate *Nf1* in the hematopoietic compartment (22). *Mx1-Cre, Nf1*$^{flox/flox}$ mice develop a MPN that closely models JMML between 5 and 6 months of age, which is characterized by hunching, an abnormal gait, and a disheveled appearance. Half of the animals die of MPN by 7.5 months. Importantly, however, this MPN does not spontaneously progress to AML (22).

Retroviral insertional mutagenesis (RIM) is a powerful strategy for generating hematologic cancers in mice and for identifying genes that contribute to leukemogenesis (23-25). We have made extensive use of MOL4070LTR, a replication competent ecotropic murine leukemia virus that induces myeloid leukemia (26). By infecting *Nf1* mutant mice with MOL4070LTR, we generated a diverse collection of primary AMLs that model the multi-step pathogenesis of advanced human cancers. Our

ability to transplant primary leukemia cells into irradiated recipient mice provides a powerful system for testing experimental agents and for elucidating mechanisms of drug response and resistance (27).

Targeted Therapies for Tumors Characterized by Hyperactive Ras Signaling. Oncogene addiction likely explains the *in vivo* therapeutic index of targeted and conventional anti-cancer agents and is particularly evident in cancers in which somatic mutations result in the production of activated kinases. However, the most common cancer-associated mutations do not encode proteins with aberrant gain-of-function biochemical activities that are as readily "drugable". For example, *NF1* inactivation abrogates neurofibromin function. The extensive evidence suggesting that elevated levels of Ras-GTP plays a central role in tumorigenesis in NF1 suggests that inhibiting activated Ras is a logical therapeutic alternative. However, Ras proteins are exceedingly difficult targets for drug discovery because of structural constraints within the phosphate-binding loop and the need to augment GTP hydrolysis rather than inhibit an over-active enzyme (28, 29). Efforts to target post-translational modifications in Ras through the use of farnesyltransferase inhibitors also failed due to the existence of alternate processing enzymes for N-Ras and K-Ras (30-32).

Given the inherent difficulties in directly targeting the Ras/GAP GTPase switch, multiple small molecule inhibitors of Ras effectors such as Raf, MEK and Akt have been developed and tested in patients with advanced cancers (**Fig. 1**). Indeed, the B-Raf inhibitor vemurafenib and the MEK inhibitor trametinib have been approved by the Food and Drug Administration for the treatment of melanomas with *BRAF* mutations An important limitation of applying this strategy to cancers with mutations in *KRAS, NRAS,* or *NF1* is that Ras-GTP activates a complex network of downstream molecules, and it is uncertain which of these effectors contribute to tumor formation and maintenance in different cell types. However, genetic analysis of human cancers demonstrating frequent somatic mutations in components of the Raf/MEK/ERK and phosphatidylinositol 3' kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) kinase effector cascades as well as studies of tumors from patients with NF1 and *Nf1* mutant mice showing aberrant activation of these pathways support the potential therapeutic benefit of PI3K, mTOR, and MEK inhibitors (14, 27, 33-36). We have executed preclinical trials in accurate mouse models of early stage and advanced hematologic cancers driven by *Nf1* inactivation or oncogenic *Kras* expression (27, 30, 37-39). Based on this experience, we are pursuing new mechanistic and translational studies to advance the long-term goal of implementing better therapies for advanced NF-associated cancers.

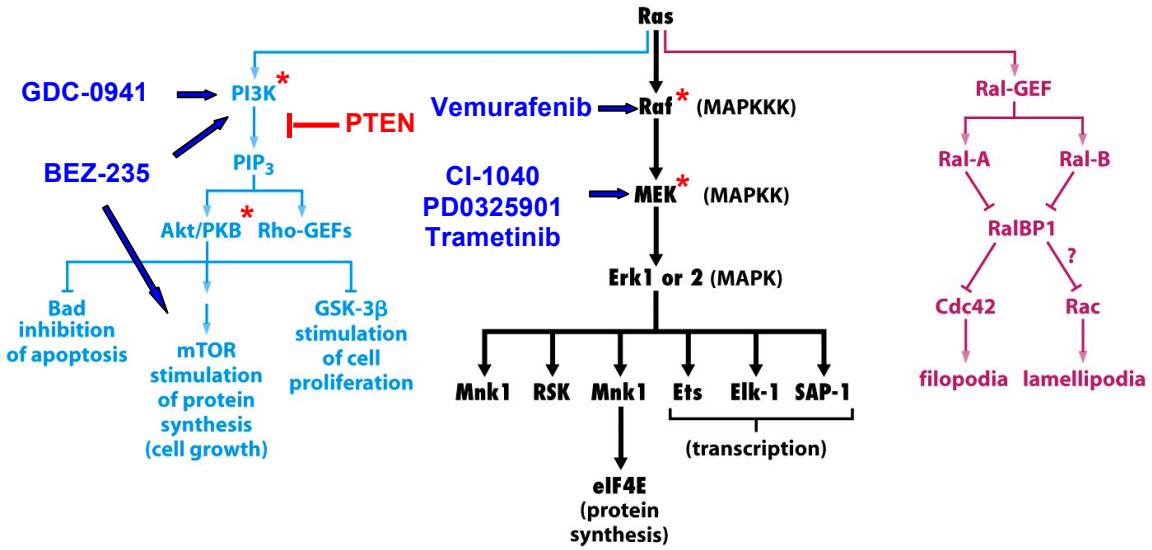

***Figure 1*. Overview of Major Ras Effector Pathways.** *Proteins that are altered by oncogenic mutations in human cancer are indicated with red asterisks and PTEN is commonly inactivated in many cancers. Small molecule inhibitors that we have tested in our preclinical models are shown in blue with arrows pointing to their targets.*

Response and Resistance to MEK Inhibition in *Mx1-Cre Nf1$^{flox/flox}$* Mice with AML. To model the progression of JMML to AML seen in human patients, we injected neonatal *Mx1-Cre, Nf1$^{flox/flox}$* mice and control *Nf1$^{flox/flox}$* littermates with MOL4070LTR retrovirus and pI-pC (27). In this screen, *Mx1-Cre, Nf1$^{flox/flox}$* mice demonstrated a higher incidence of AML as well as reduced latency. These AMLs are biologically aggressive and are readily transplantable into recipient mice given a sublethal dose of irradiation (450 cGy).

We obtained CI-1040, a "first generation" MEK inhibitor, from Pfizer, Inc., and found that 25-50 μM of CI-1040 abrogated CFU-GM colony formation from *Mx1-Cre, Nf1$^{flox/flox}$* bone marrow. Importantly, however, there was no therapeutic index as CFU-GM growth from wild-type (WT) bone marrow was inhibited at similar concentrations (27). By contrast, blast colony growth from *Mx1-Cre Nf1$^{flox/flox}$* AML bone marrow was abrogated at much lower drug concentrations of CI-1040. These unexpected data suggested that mutations that are acquired during progression from MPN to AML make leukemic cells more dependent on Raf/MEK/ERK signaling. To test this hypothesis *in vivo*, we transplanted *Nf1* mutant AMLs and treated the recipients with CI-1040 or with PD0325901 (PD901) a "second generation" MEK inhibitor with enhanced pharmacokinetic properties (27). Both drugs induced clinical remissions and markedly prolonged survival. However, all of the mice eventually relapsed and died of AML despite continued treatment. Resistant leukemias were remarkably less sensitive to MEK inhibitors *in vitro* than the corresponding parental AMLs, and did not respond to treatment in secondary recipients. Drug resistance was not due to acquired *Mek1* mutations, and MEK remained sensitive to biochemical inhibition by CI-1040 or PD901.

Importantly, analysis of clinical evolution by Southern blotting revealed recurrent novel retroviral integrations in three resistant clones (6537R, 6554R1, and 6554R2) that emerged in multiple independent recipient mice transplanted with primary AMLs 6537 and 6554. This observation provided compelling evidence that the resistant clones were present at undetectable levels in the primary AML, and is consistent with recent studies of human leukemia (40-43). We exploited a shotgun cloning strategy (44) to identify novel retroviral integrations in AMLs 6537R, 6554R1, and 6554R2. This analysis implicated p38α and guanine nucleotide exchange factors of the Ras-GRP family in resistance to MEK inhibitors, which we went on to functionally validate. These studies provide "proof of principle" that *in vivo* treatment with targeted agents followed by molecular analysis of paired sensitive/resistant leukemias is a potent and unbiased strategy for monitoring clonal evolution in response to targeted anti-cancer agents and for uncovering genes that underlie "off target" resistance (27). We are deploying these methodologies and novel reagents to pursue the aims of this application.

Progress Report

Technical Objective (Aim 1): Restoring neurofibromin GAP activity in primary AMLs

Primary *Nf1* mutant AMLs are transplantable into sublethally irradiated WT mice, and we exploited this property to perform the preclinical studies of targeted inhibitors described above. As summarized in our 2013 Progress Report, the ability to manipulate AML cells *ex vivo* before transplanting them provides a potential opportunity to ask how restoring neurofibromin GAP activity modulated the proliferation and survival of primary *Nf1*-deficeint cancer cells. Because this approach is technically challenging, we first focused on developing and validating a lentiviral delivery system for modulating gene expression in human AML cell lines with somatic *NRAS* mutations and in primary murine AMLs generated in *Nras* mutant mice (45). Advantages of this initial approach include the small size of the *NRAS/Nras* genes and the availability of excellent N-Ras antibodies. In data presented in our 2013 Progress Report, we first showed that two independent shRNA constructs specific for human *NRAS* inhibited the growth 3 *NRAS* mutant AML cell lines, but had no effect on AML cells with other "driver" mutations in genes encoding signaling molecules *(e.g. KRAS, FLT3).* In subsequent studies,

we engineered this vector to express shRNAs against murine *Nras* and infected primary AML cells. These experiments revealed remarkable inhibition of leukemia growth *in vivo* that was selective for *Nras* mutant AML cells. Together, these studies performed in year 1 of this project generated "proof of concept" data for extending this general approach to re-express the neurofibromin GAP domain in primary AMLs driven by loss of *Nf1*.

In studies performed during this reporting period, we designed lentiviral vectors expressing either the wild-type neurofibromin GAP related domain (GRD) or a mutant GRD containing a R1276P amino acid substitution. The R1276P mutation alters a critical "Arg finger" residue that is essential for accelerating Ras GTPase activity. Importantly, the substitution was reported in a family with classic NF1 and was previously shown to markedly impair GAP activity (46). We engineered these constructs to encode a N-terminal hemagglutinin (HA) tag that can be used to directly assess protein expression by Western blotting and for immunoprecipitation experiments to measure GAP activity in transduced cells using a ^{32}P release assay from labeled recombinant Ras-GTP (47, 48). We also cloned a C-terminal "self-cleaving" T2A peptide into this vector followed by genes encoding either green (GFP) or red (mCherry) fluorescent proteins for monitoring expression of the GRD in living cells (**Fig. 2a**). Utilization of the T2A peptide cleavage site establishes an internal reference that insures that the measured fluorescence is directly proportional to the level of GRD expression *in vivo*.

We confirmed expression of the HA-tagged GRD and GRD R1276P proteins by Western blot in NIH 3T3 cells (**Fig. 2b**). Interestingly, multiple experiments performed in different cell contexts suggest that high levels of the wild-type GRD expression are not tolerated presumably due to super-physiologic levels of GAP activity. By contrast, transduced cells express high levels of the mutant HA-GRD R1276P. To test the functional activity of the GAP construct in a disease-relevant context, we overexpressed the HA-GRD and mutant HA-GRD R1276P in a panel of human AML cell lines harboring oncogenic *FLT3* (MOLM-14 and MV-11) or *NRAS* (OCI-AML3 and THP-1) mutations. Following infection, we sorted GFP positive cells to purity, plated cells at equal density, and assessed

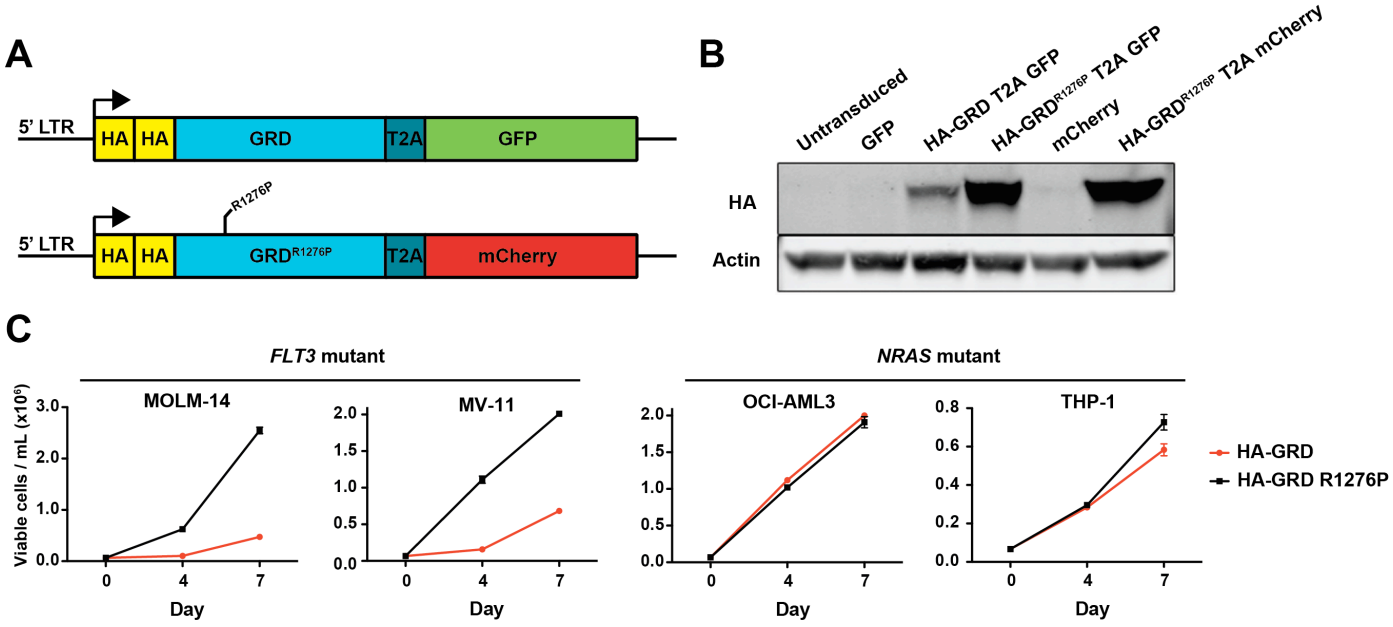

Figure 2*. Expression of the neurofibromin GAP related domain (GRD) and "Arg finger" R1276P mutant for assessing dependence on GAP activity.** ***A. *Lentiviral constructs designed to express the N-terminal HA epitope tagged wild-type GRD or the R1276P mutant with C-terminal self-cleaving T2A peptide followed by a fluorescent protein.* ***B.*** *Western blot for HA demonstrating expression of the HA-GRD and R1276P mutant in NIH 3T3 cells.* ***C.*** *Overexpression of HA-GRD in FLT3 mutant human AML cell lines dramatically attenuates growth compared with expression of the control R1276P mutant lacking GAP activity. Human AML cell lines harboring oncogenic NRAS mutations are resistant to increased GAP expression.*

cell viability after 4 and 7 days of growth in liquid culture (**Fig. 2c**). Human AML cell lines harboring *NRAS* mutations were insensitive to exogenous GRD expression, which is consistent with the known resistance of oncogenic N-Ras proteins to GAPs. By contrast, AML cells with activating mutations in *FLT3,* which encodes a receptor tyrosine kinase that activated Ras, showed reduced growth in cells expressing the HA-GRD construct (**Fig. 2c**).

We next infected mouse *Nf1*-deficient AML 6537 (27) with lentiviral vectors encoding GFP only, HA-GRD T2A GFP, or HA-GRD R1276P T2A GFP, and sorted GFP-positive (GFP^+) cells to purity after 2 days of growth in liquid culture. To test whether exogenous HA-GRD expression altered growth factor signaling, we starved GFP^+ cells for 3 hours, stimulated them with 10 ng/mL of GM-CSF for 15 minutes, and assessed ERK phosphorylation by flow cytometry as described previously (38, 39). AML cells expressing HA-GRD showed attenuated ERK activation compared with the same 6537 leukemia infected with empty vector or HA-GRD R1276P (**Fig. 3a**). We also plated GFP^+ cells in methylcellulose medium and assessed AML blast colony formation in the absence or presence of a saturating concentration of GM-CSF. Expressing HA-GRD dramatically attenuated colony formation by $Nf1^{-/-}$ AML 6537, which was rescued by a saturating concentration of GM-CSF (**Fig. 3b**). These data suggest that *Nf1* loss is required for leukemia maintenance in AML 6537.

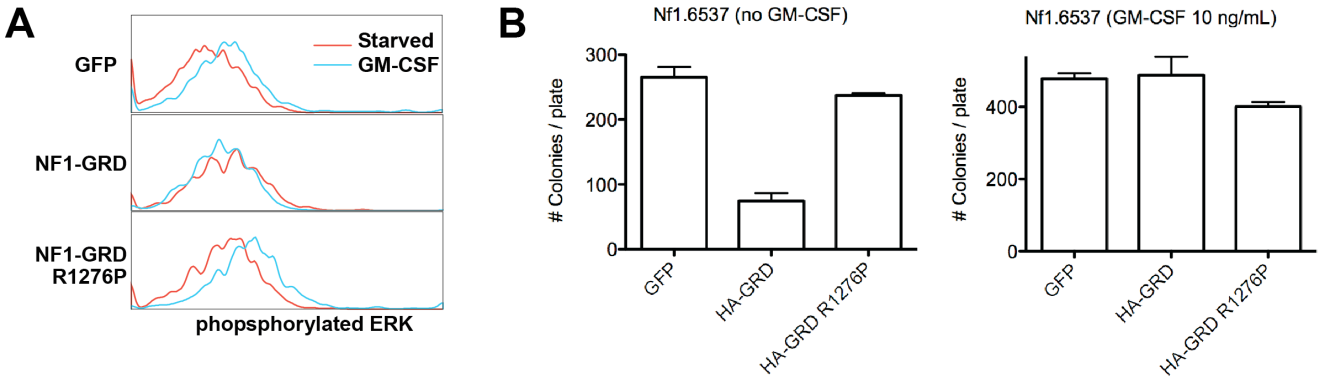

***Figure 3**. **Restoring GAP activity in primary murine AMLs characterized by Nf1 inactivation.** **A.** Phospho-FACS depicting $Nf1^{-/-}$ AML 6537 infected with lentiviral constructs, sorted to purity, starved, and stimulated with 10 ng/mL GM-CSF. ERK phosphorylation is attenuated with overexpression of HA-GRD but not the empty vector or the HA-GRD R1276P control. **B.** Methylcellulose blast colony assays for $Nf1^{-/-}$ AML 6537 infected with the corresponding lentiviral construct in the absence or presence of saturating GM-CSF.*

We are currently deploying this tractable system for restoring GAP activity in primary *Nf1*-deficient cancer cells if this attenuates the growth of *Nf1* mutant AMLs 6537 and 6554 *in vivo*. These studies will be performed under competitive repopulation conditions using primary AMLs 6537 and 6554 cells transduced with constructs expressing HA-GRD T2A GFP or HA-GRD R1276P T2A mCherry (**Fig. 2**), mixing the cells at a 50:50 ratio, and transplanting them into recipient mice. We will compare the percentage of GFP and mCherry-expressing cells at the time of transplantation and at of the recipients death from leukemia. As a control, we will infect parental AMLs with HA-GRD R1276P T2A GFP or mCherry and transplant 50:50 mixes. Based on the blast colony assay data shown in Figure 3, we hypothesize that AML cells expressing the wild-type GRD will be out-competed by cells expressing either mutant HA-GRD R1276P or mCherry. In addition, we will measure the median fluorescence at transplant and at the time of euthanasia, as this is a surrogate marker of GRD protein expression. We predict that any leukemia cells transduced with HA-GRD that survive *in vivo* will show markedly reduced GFP expression relative to the HA-GRD R1276P control.

One caveat of the current lentiviral transduction system is that we cannot entirely exclude the possibility that over-expressing the GRD attenuates leukemia engraftment rather than affecting disease maintenance. Furthermore, because lentiviral transduction of the primary mouse AMLs requires short-term culture, it is possible that over-expressing the GRD induces physiologic changes before transplantation. Consistent with this idea, we have noted both reduced transduction efficiency and reduced expression as measured by median fluorescence in cells transduced with HA-GRD after short-term culture (data not shown). For this reason, we are working to establish a tetracycline-controlled transcriptional activation system by utilizing one lentiviral construct engineered to express the Tet-On transactivator (rtTA) with a blue fluorescence protein (BFP) and a second construct that expresses HA-GRD T2A GFP under the control of a Tet-On promoter with constitutive mCherry expression from the PGK promoter. We have effectively utilized this system in mouse hematopoietic cell lines where we have demonstrated robust induction of GFP after 36 hours of doxycycline treatment with no background in the absence of doxycycline (data not shown). We plan to co-infect primary mouse AMLs with rtTA BFP and the HA-GRD and HA-GRD R1276P mutant, sort to purity, and transplant secondary recipients with BFP and mCherry double-positive cells. We will monitor recipients for engraftment by measuring the appearance of mCherry-positive blasts in peripheral blood. We will then plan to treat the mice with doxycycline and assess survival.

We hypothesize that the survival of mice transplanted with AML cells expressing the wild-type GRD domain will be greatly prolonged compared to recipients transplanted with the same AML cells expressing either the control GFP vector or a R1276P mutant GRD. We will also assess GRD expression and biochemical GAP activity in recipients that develop AML after receiving cells expressing the wild-type GRD to ask if they no longer express this protein. Finally, it would be interesting to see if the pattern of retroviral integrations changes in AMLs with restored wild-type GRD expression. After we characterize the *in vivo* effects of restored GAP expression in the parental *Nf1* mutant AMLs 6537 and 6554, we will then perform a similar analysis by transducing and transplanting the three resistant subclones (6537R, 6554R1, and 6554R2) with our inducible HA-GRD and HA-DRG R1276P vectors. It will be particularly interesting to determine if MEK-resistant subclones differ from the respective parental AMLs with respect to GRD dependence, or if these leukemia cells are no longer require *Nf1* inactivation for survival and proliferation *in vivo*.

In addition to studying the functional consequences of restoring wild-type GRD expression in primary mouse leukemias, we hope to utilize this system as a tool to study the regulation of neurofibromin function. For example, Spred1 (Sprouty-related protein with an EVH1 domain) functions as a negative regulator of the Ras/Raf/MEK/ERK pathway by binding neurofibromin and mediating translocation to the plasma membrane where it can perform its function as a Ras GAP in regulating growth factor receptor signaling (49). Deletion of GRD amino acid M1215 abrogates the interaction with Spred1 while retaining full biochemical Ras GAP activity. We plan to over-express HA-GRD delM1215 in our panel of primary *Nf1* mutant mouse leukemias to ask if this attenuates blast colony formation or leukemia growth *in vivo* compared with wild-type GRD. These studies will address if membrane recruitment by Spred1 is required for the tumor suppressor activity of neurofibromin in primary leukemia cells. Given our recent success in generating and validating HA-GRD T2A GFP and HA-GRD R1276P T2A mCherry vectors, we anticipate no difficulties in carrying out these studies during the coming fund year.

Technical Objective (Aim 2): Preclinical studies combining cytotoxic chemotherapy with MEK inhibitors in primary murine AMLs.

Our preclinical studies in *Nf1* mutant mice and observations in patients treated with small molecule inhibitors of signaling molecules showed that many advanced cancers respond transiently before becoming resistant (27, 50). Emerging data also suggests that combining targeted and conventional

chemotherapeutic agents increases clinical efficacy (51), and genetically diverse transplantable *Nf1* mutant AMLs provide a robust system for applying this principle to NF1-associated cancers.

The standard treatment for AML involves administering high doses of cytarabine in combination with an anthracycline agent such as daunorubicin. These aggressive regimens induce remissions in ~80% of patients who are <60 years old; however, over half ultimately relapse despite receiving consolidation chemotherapy and/or HSCT. CPX-351 is a liposomal formulation that contains cytarabine and daunorubicin at an optimized ratio to enhance AML killing without added toxicity (52). CPX-351 in being investigated in human clinical trials, and dosing schedules were developed for treating immunodeficient mice engrafted with human leukemia cell lines (53, 54). Based on the "front line" role of cytarabine:daunorubicin in human AML treatment protocols and our data demonstrating murine AMLs that have inactivated the *Nf1* gene are sensitive to MEK inhibitors *in vitro* and *in vivo,* we initiated preclinical trials to begin testing the hypothesis that combining these drugs will have synergistic effects.

As summarized in our 2013 Progress Report, we first sought to establish the maximally tolerated dose (MTD) dose of CPX-351 in mice treated with subletal irradiaiton and transplanted with primary murine AML cells. Immunodeficient mice engrafted with a human leukemia cell line tolerated a regimen that administered CPX-351 at a cytarabine:daunorubicin ratio of 10 mg:4.4 mg on days 1, 3, and 5 (induction phase) followed by 50% of the induction dose on days 21 and 26 (consolidation phase)(52, 54). Our initial studies proved that these doses were too toxic in our model as all recipient mice died shortly after the end of induction. Based on discussions with the scientific team at Celator, the induction dose was decreased and fixed at 5 mg:2.2 mg, a clinically relevant dose that maintains anti-leukemic activity in humans. To establish the tolerability of this regimen with the conditioning sublethal radiation required for leukemia engraftment in transplant recipients, we sublethally irradiated WT mice on day zero and injected them with three 5 mg:2.2 mg doses of CPX-351 beginning four days later (days +4, +6, and +8). We monitored these mice for toxicity by weekly weights and by performing complete blood counts to monitor for myelosuppression. We determined that CPX-351 at 5 mg:2.2 mg for a total of three doses was well tolerated with pronounced leukopenia in CPX-351 treated animals two weeks following radiation followed by hematologic recovery by day 21 similar to control radiated animals (**Fig. 4a**). The degree of anemia was similar in both groups.

To determine the efficacy of CPX-351 in primary murine AMLs, we treated recipeint mice that were tranplanted with a panel of *Nf1* mutant (n=1), $Kras^{G12D}$ (n=1), and $Nras^{G12D}$ (n=3) AML cells with CPX-351 using the schedule outlined above. We observed a modest improvement in overall survival that did not reach statistical significance (p=0.0746; **Fig. 4b**). Despite this, there were individual leukemias that responded to therapy with CPX-351 including $Kras^{G12D}$ AML 101 and *Nf1* mutant AML 6537 (**Fig. 4c, 4d**). Given the dramatic improvement in survival in both of these AMLs to single-agent PD901 (27); and data not shown), we tested the combined effects of cytotoxic therapy with CPX-351 followed by PD901 consolidation. In our initial trial, we started PD901 14 days after the last dose of CPX-351. Unfortunately this dosing sechedule proved too toxic, as the majority of animals succumbed with bone marrow failure shortly after starting the MEK inhibitor (**Fig. 4b**). We interrogated these AMLs for evidence of clonal evolution and did not observe changes at the level of retroviral integration with this dosing schedule (**Fig. 4e**). Intriguingly, there was one animal transplanted with $Kras^{G12D}$ AML 101 that had markedly prolonged survival suggesting CPX-351 induction followed by PD901 consolidation may be beneficial with a dosing schedule that is more tolerable (**Fig. 4c**). The observed toxicity is not unexpected as irradiation, leukemic marrow infiltration, cytotoxic drugs, and PD901 all contribute to bone marrow suppression.

In summary, we are encouraged by the benefit in survival observed with CPX-351 and hope that a modified dosing schedule will allow us to study the combined effects of MEK inhibition and cytotoxic chemotherapy in AML. We are currently adjusting the PD901 dosing schedule to begin day

21 after CPX-351, and are also extending the duration of CPX-351 induction to increase tolerability of this regimen.

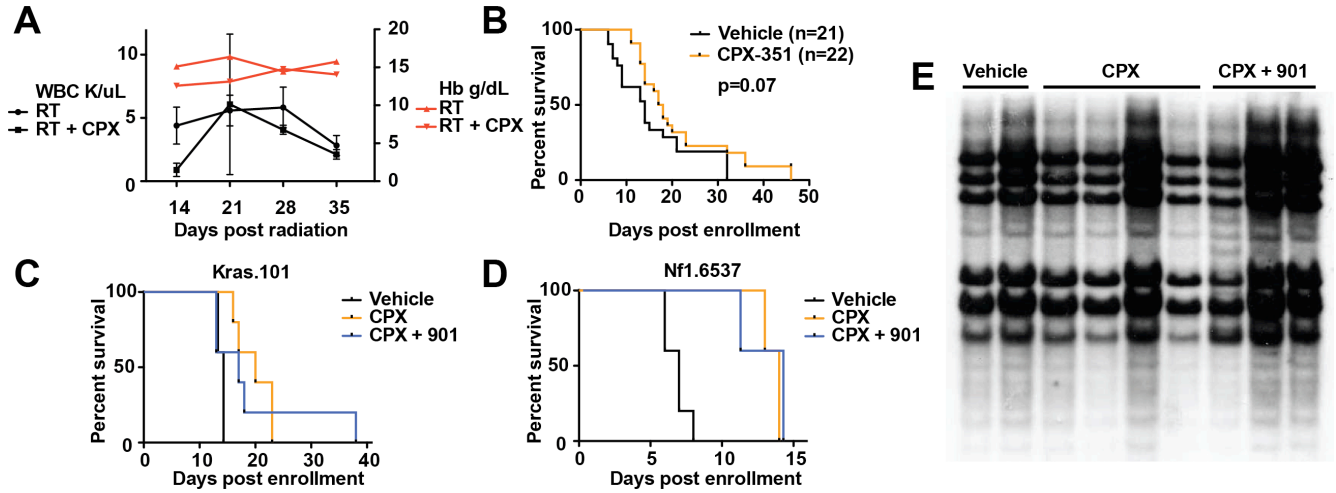

Figure 4. Treatment of primary murine AMLs with CPX-351 (CPX) alone and in combination with PD0325901 (901). ***A.*** *Tolerability of CPX-351 following sublethal conditioning radiation therapy (RT) required for leukemic engraftment in wild-type mice. Weekly mean white blood cell count (WBC) is shown in black and mean hemoglobin (Hb) is shown in red.* ***B.*** *Kaplan-Meier analysis demonstrates improved survival in mice assigned to treatment with CPX-351 (orange line) compared to recipients transplanted with the same leukemias that received control vehicle (black line) though the aggregate analysis of these five leukemias did not reach statistical significance.* ***C.*** *Individual leukemias including Kras^{G12D} AML 101, and* ***D.*** *Nf1 mutant AML 6537 showed prolonged survival with CPX-351; however, mice assigned to combination therapy with CPX and 901 suffered early mortality secondary to bone marrow suppression.* ***E.*** *CPX-351 failed to induce clonal evolution at the level of retroviral integrations in this model with a representative Southern blot from Kras^{G12D} AML 101 shown.*

One additional question in combining cytotoxic chemotherapy and MEK inhibition is how the order is which these agents are given influences the emergence of drug resistant disease. We have generated 5 independent MEK inhibitor resistant AML clones with *in vivo* drug selection including three leukemias with inactivation of *Nf1* (AMLs 6537-R, 6554-R1, and 6554-R2)(27), and two *Kras^{G12D}* leukemias (*Kras^{G12D}* AMLs 101-R and 21B-R). As is often true of relapsed human AML, resistant *Nf1* and *Kras* AMLs exhibit more aggressive biologic properties than the corresponding parental AML. For example, AML 101-R induces a shorter time to death in untreated recipients (12 versus 15 days), has a shorter doubling time in liquid culture, and causes a more significant leukocytosis and splenomegaly in recipients (**Fig. 5a, 5b**). We sought to determine how CPX-351 might differentially influence the fitness of MEK inhibitor sensitive and resistant clones as this may suggest how to combine these agents for enhanced efficacy.

In a pilot experiment, we used lentiviral vectors to fluorescently labeled AML 101 with mCherry and GFP and treated AML 101-GFP with PD901 to derive drug resistant GFP-labeled clones. We then injected secondary recipients with a mix of 10% GFP-labeled PD901-sensitive or resistant leukemia and 90% control parental mCherry leukemia. Measuring the amount of GFP versus mCherry labeled AML cells in untreated recipients at the time of death showed that the PD901 resistant AML 101-R clone dramatically outcompetes the parental leukemia (4.5-fold ±0.6) even in the absence of PD901 demonstrating increased fitness *in vivo* of the drug resistant clone (**Fig. 5c**). Next, we asked whether CPX-351 could differentially affect the growth of the PD901 resistant clone. We transplanted recipient mice with a 90:10 mix of mCherry-positive parental leukemia and GFP-labeled PD901 resistant or control AML cells. On day 14, the PD901 resistant clone significantly out-competed the parental leukemia (**Fig. 5d, left**). We then serially passaged these bone marrow cells into multiple recipient mice and treated them with vehicle, PD901, or CPX-351 (**Fig. 5d, right**). In secondary recipients, the AML 101-R-GFP clone continued to out-compete the parental AML with an enhanced

competitive advantage with PD901 treatment. In contrast, CPX-351 dramatically suppressed the PD901 resistant clone relative to the parental leukemia suggesting increased sensitivity to this cytotoxic agent. These data demonstrate that we have implemented a tractable system for *in vivo* modeling of the clonal dynamics of drug sensitive/resistant leukemia populations and can directly measure how cytotoxic chemotherapy influence the outgrowth of drug resistant populations.

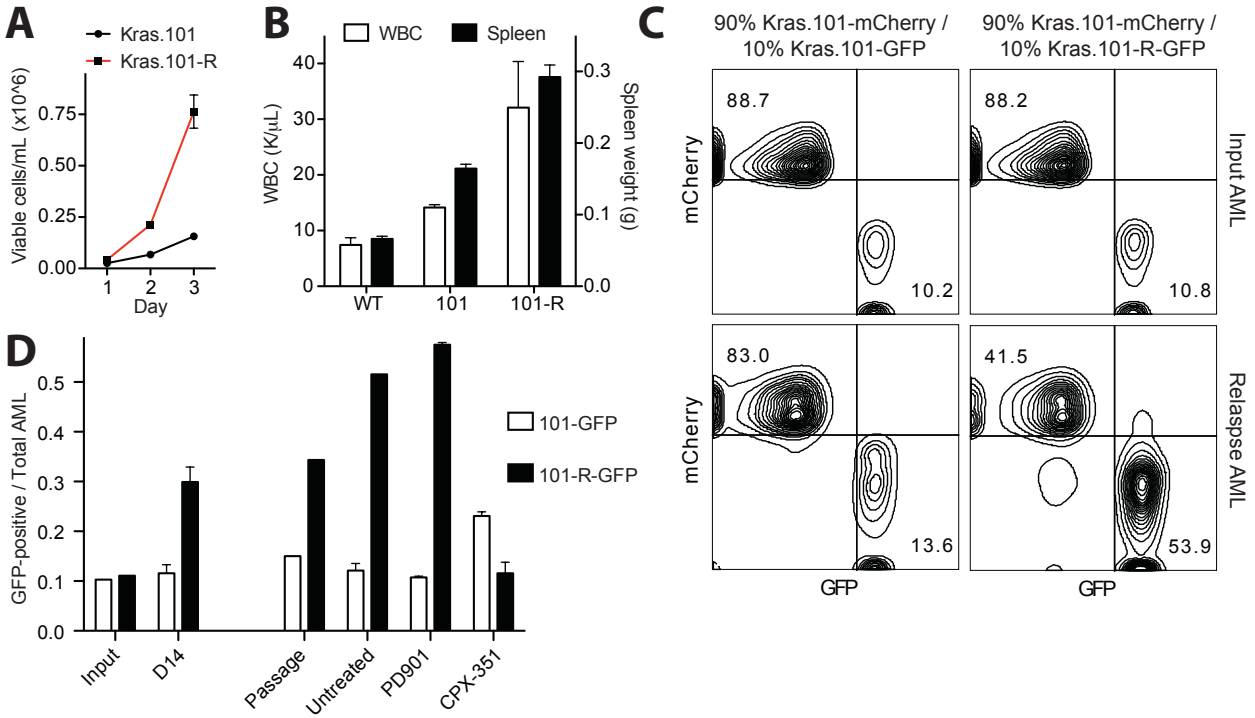

***Figure 5.* CPX-351 modulates the fitness of MEK inhibitor resistant clones in vivo.** *PD901 resistant 101-R clone is more aggressive than the parental leukemia with (A) increased proliferation in liquid culture and (B) more pronounced leukocytosis (white) and splenomegaly (black) in secondary transplant recipients one week post-transplant. (C) AML 101-mCherry parental leukemia was mixed with 10% GFP-labeled parental (left) or 101-R (right) and transplanted into secondary recipients. We measured the percentage of mCherry and GFP-labeled cells by FACS at input (top) and isolated from whole bone marrow at death (bottom) with representative samples shown. We observed a 4.5-fold (±0.6) increase in the 101-R-GFP clone (bottom right). (D) Following competitive repopulation as above, transplant recipients were euthanized at day 14 (left) and passaged into a cohort of recipient animals that were treated with vehicle, PD901, or CPX-351. The contribution of GFP-positive/total leukemia was measured by FACS after drug exposure (right).*

Based on these data, we are actively testing how the sequence of cytotoxic chemotherapy with CPX-351 and MEK inhibition with PD901 or GSK1120212 (trametinib) may influence the fitness of drug resistant AML clones. We have generated fluorescently labeled *Nf1* mutant AML drug sensitive/resistant pairs and are conducting similar experiments to test the fitness of drug resistant clones under the selective pressure of cytotoxic therapy as above (**Fig. 5**). We can directly ask whether the MEK inhibitor first, CPX-351 first, or concurrent treatment has a more pronounced effect on suppressing the drug resistant clones or overall survival. The goal of these studies is to develop a preclinical rationale for therapeutic approaches aimed at the suppression of drug resistant disease during therapy. We anticipate these studies will inform the design of clinical trials employing cytotoxic therapy and MEK inhibition particularly in NF1 patients.

Preclinical evaluation of MEK inhibitor Trametinib in Nf1 Mutant Mice with MPN

PD901 and trametinib (GSK1120212; GSK) are chemically related allosteric inhibitors of MEK. Despite this, there are compelling theoretical and practical reasons to fully evaluate trametinib

in mouse models of NF1-associated cancers. First, whereas PD901 has excellent central nervous system (CNS) penetration in animals (55), trametinib does not accumulate in the brain (56). Since many NF1-associated cancers affect, minimizing potential CNS complications is highly desirable. Second, trametinib is regarded at "best in class" among the current generation of MEK inhibitors, and is approved by the FDA for the treatment of advanced melanoma. Finally, GSK has an active clinical development plan and timeline for trametinib in pediatric patients (including children with NF1), and has a liquid formulation available, which is not true for PD901.

To accelerate clinical translation, we first evaluated trametinib in *Mx1-Cre, Nf1$^{flox/flox}$* mice with MPN. These studies are not part of the scientific goals of this CDMRP-funded project, and were funded by a grant from the Children's Tumor Foundation. Our results are summarized here because they are directly relevant to testing the most promising drug combinations in AML and other NF1-assocaited cancers. As described above, we will utilize data from our studies of mice with MPN to develop preclinical trials to investigate the efficacy of CPX-351 + trametinib.

We first performed pharmacodynamic studies to determine the lowest dose of trametinib that achieves durable target inhibition *in vivo.* Congenic wild-type (WT) mice that received trametinib for 5 days at a dose of 0.5 or 1.0 mg/kg/day were euthanized, and bone marrow was harvested to assess target inhibition 6, 12, and 24 hours after treatment. We also collected blood by cardiac puncture from mice 2, 4, 8, and 24 hours after dosing, and shipped frozen plasma to GSK for pharmacokinetic (PK) studies. Pharmacodyamic (PD) analysis of bone marrow revealed sustained inhibition of GM-CSF-stimulated ERK phosphorylation at both dose levels that persisted at the 24 hour time point **(Figs. 6A, 6B)**. This duration of target inhibition after 0.5 mg/kg/day of trametinib was equivalent to what we achieved in mice treated with PD901 at 5 mg/kg/day (27, 38, 39). PK analysis revealed a peak drug concentration 8 hours after dosing with measurable levels of drug in plasma after 24 hours (**Fig. 6C**).

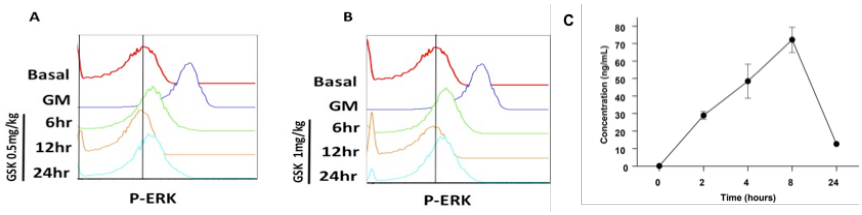

Figure 6. Trametinib (GSK) inhibits MEK in vivo. *Mice were treated with 0.5 mg/kg/day* ***(A)*** *or 1 mg/kg/day* ***(B)*** *for 5 days. P-ERK levels were measured by flow cytometry in Mac1^{+} Gr1^{+} bone marrow cells after stimulation with a saturating dose of GM-CSF (10ng/mL). Both doses achieve comparable inhibition of ERK phosphorylation.* ***C.*** *PK analysis of plasma trametinib concentrations in the mice shown in panel B. The data in panel C were provided by Jessica Gannon (GSK).*

We treated congenic WT mice with either 0.5 mg/kg/day or 1 mg/kg/day of trametinib for 6 weeks. These mice remained well with no adverse effects of treatment. Based on the PK and PD data shown in Figure 6, we performed a pilot efficacy trial at the 0.5 mg/kg/day dose. *Mx1-Cre; Nf1$^{flox/flox}$* and control WT mice were enrolled at 5-9 months of age, and randomized to receive treatment with trametinib or vehicle (n= 4-5 in each arm). After 8 weeks, some drug-treated mice developed a dry erythematous rash that first appeared in the oldest animals, and preferentially affected *Nf1* mutant mice. We terminated the trial and analyzed hematologic responses. Treatment with trametinib resulted in marked improvements in blood leukocyte counts and spleen weights in *Nf1* mutant mice with MPN (**Figs. 7A-D**).

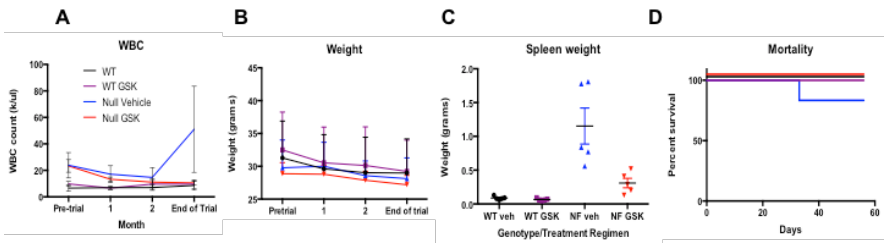

Figure 7. Response of Mx1-Cre, Nf1$^{flox/flox}$ mice to trametinib (GSK) or control vehicle at 0.5 mg/kg. *In all panels, Mx1-Cre, Nf1$^{flox/flox}$ mice with MPN treated with control vehicle are shown in blue; NF$^{flox/flox}$ mice treated with trametinib are in red; WT mice given the vehicle are in black; and WT mice treated with trametinib are in purple (n=4-6 in each cohort).* ***A.*** *Blood leukocyte counts.* ***B.*** *Weight.* ***C.*** *Spleen weight at the end of treatment.* ***D.*** *Survival through the end of 8 weeks of treatment.*

Erythropoiesis was also markedly improved after 8 weeks of trametinib, with the numbers of erythroid colony and burst forming unit (CFU-E and BFU-E) progenitors in the spleen returning to near-normal levels in *Nf1* mutant mice with MPN (**Fig. 8A).** Similarly, treatment with trametinib reduced the numbers of infiltrating early (c-kit^{+}, lin^{-}, sca1^{+}; KLS) and differentiated myeloid progenitors (**Fig. 8B**). These responses were quantitatively and qualitatively similar to what we observed in *Nf1* mutant mice treated with 5 mg/kg/day of PD901 (39).

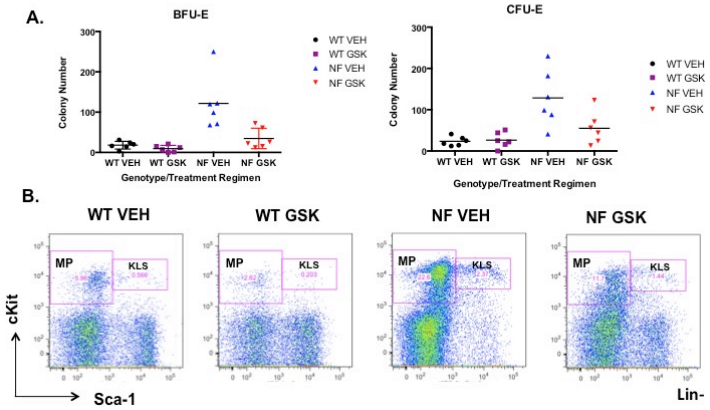

Figure 8. Treatment with trametinib (GSK) reduces splenic infiltration by erythroid and myeloid progenitors in Mx1-Cre, Nf1$^{flox/flox}$ mice. ***A.*** *Numbers of BFU-E and CFU-E colonies grown from splenocytes of WT and Nf1 mice treated with vehicle or trametinib (GSK) for 8 weeks.* ***B.*** *Representative profiles of myeloid progenitors (MP) and KLS cells in the spleens of WT and Nf1 mice treated with vehicle or trametinib for 8 weeks.*

Technical Objective (Aim 3): Identify and validate resistance genes in Nf1 mutant AML

These studies are dependent on generating *Nf1* mutant AMLs that respond to CPX-351 as a single agent or in combination with a MEK inhibitor (PD901 or trametinib), but subsequently relapse. We have not yet isolated any leukemias that fulfill these criteria, but are optimistic that these efforts will be successful based on progress made to date and our expectation that we will develop combination regimens that will bypass the cumulative toxicity observed to date in mice given CPX-351 + PD901 or trametinib.

KEY RESEARCH ACCOMPLISHMENTS

(a) We utilized a lentiviral transduction/transplantation system to investigate how restoring neurofibromin GAP activity (GRD) modulates the growth of primary *Nf1* mutant AMLs. These preliminary studies support the hypothesis that at least some advanced cancers remain dependent on *Nf1* inactivation for growth. To address this fundamental question with greater precision, we are developing a tetracycline-controlled transcriptional activation system to overcome some of the technical limitations of constitutive GAP overexpression in primary AML cells. We will also investigate whether loss of Spred1 binding modulates the ability of the GRD to suppress AML growth *in vivo*. These studies will determine whether the MEK inhibitor resistant AMLs have bypassed the requirement for neurofibromin GAP inactivation.

(b) We established a tolerable dose of CPX-351 in our background strain. We treated a panel of primary murine AMLs characterized by hyperactive Ras signaling (*Nf1*, *Kras*, and *Nras* mutant) and demonstrated a modest increase in overall survival. Initial trials combining CPX-351 and PD901 were characterized by pronounced toxicity, but there is a signal that modification of the dosing regimen may lead to enhanced efficacy. Intriguingly, we have shown that CPX-351 can differentially suppress the fitness of some MEK inhibitor resistant AML clones *in vivo*.

(c) We found that a daily dose of 0.5 mg/kg/day of the MEK inhibitor trametinib results in excellent

pharmacodynamic target inhibition in bone marrow cells and is efficacious in *Nf1* mutant mice with MPN. We will incorporate trametinib into our preclinical AML testing program in the next fund year.

REPORTABLE OUTCOMES

(a) Research Articles & Reviews

Burgess MR, Hwang E, Firestone AJ, Xu J, Bohin N, Wen T, Haigis KM, Shannon K, Li Q. Preclinical efficacy of MEK inhibition in *Nras* mutant acute myeloid leukemia. (submitted).

(b) Abstracts

None to date.

(c) Funding applied for based on work supported by this award

Research Grant and Consortium Grant in Pediatric Cancer Research: Bear Necessities and Rally Foundation (Kevin Shannon, MD)- awarded

American Cancer Society Postdoctoral Fellowship (Michael Burgess, MD, PhD) - awarded

(d) Employment and research opportunities

This award has provided salary support for technical personnel in the lab and has facilitated the training of two talented physician/scientists in the lab (Michael Burgess, MD, PhD and Tannie Huang, MD).

CONCLUSIONS

As summarized in the "Key Research Accomplishments" section above, we have made substantial progress toward achieving the goals of this project. To facilitate the goals of Aim 1, we developed and validated a lentiviral transduction/transplantation system for manipulating the expression of genes that impact the growth of AML cells *in vivo,* and have assessed the effects of restoring neurofibromin GAP activity in primary *Nf1* mutant AML cells. Our *in vitro* data support the hypothesis that some (and perhaps all) advanced cancers remain dependent on *Nf1* inactivation for growth. To achieve the goals of Aim 2, we established a tolerable dose of CPX-351 in our background strain. Preliminary data showing that treatment with CPX-351 prolongs survival of recipient mice transplanted with *Nf1* and *Kras* mutant AMLs suggests that we will successfully isolate mutant subclones from mice given treated with drug combinations for molecular analysis as proposed in Aim 3. In work not proposed in our original Aims that was supported by other funds, we determined a dose of trametinib that results in sustained target inhibition and showed this it is efficacious in *Nf1* mutant mice with MPN. Data obtained to date support the overall conclusion that the three aims of this project are achievable and will generate biologic and preclinical data that will both increase our understanding of drug response and resistance in NF1-associated cancers and inform the design of human clinical trials.

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