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Award Number: W81XWH-12-1-0157

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

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REPORT DATE: April 2013

TYPE OF REPORT: Annual Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE April 2013		2. REPORT TYPE Annual Report		3. DATES COVERED 1 April 2012- 31 March 2013	
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) Kevin Shannon, MD E-Mail: shannonk@pedsf.ucsf.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, San Francisco San Francisco, CA 94143				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 <i>NF1</i> 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 <i>NF1</i> loss. We have extensively characterized MPN and AML in <i>Nf1</i> mutant mice and have investigated mechanisms of drug responses and resistance. Our studies of MEK inhibitors in <i>Nf1</i> 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 <i>in vivo</i> . 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 <i>Nf1</i> inactivation in cancer progression, to develop a preclinical paradigm for combining conventional and targeted anti-cancer agents <i>in vivo</i> , 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 <i>in vivo</i> , have shown that treatment with CPX-351 prolongs survival of <i>Nf1</i> mice, and have established an effective dose of the FDA-approved MEK inhibitor GSK1120212 (trametinib)					
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			Scott Linton, Technical Editor
U	U	U	UU	14	19b. TELEPHONE NUMBER (include area code) 301-619-0523

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INTRODUCTION

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

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 cases of JMML or AML that arise children with NF1. These AMLs are aggressive cancers 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 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 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. These observations support testing MEK inhibitors as single agents in JMML and in histologically benign human NF1-associated tumors such as plexiform neurofibroma. On the other hand, our preclinical data in *Nf1* mutant mice with AML and the results of human clinical trials of tyrosine kinase inhibitors in other advanced cancers indicate that drug resistance emerges rapidly. These data suggest that that administering different targeted inhibitors to simultaneously inhibit multiple signaling pathways and/or combining targeted and conventional cytotoxic drugs will be required to cure 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 future 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 malignant neoplasms 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 appear to be 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 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 that arise 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. *Mx1-Cre, Nf1^{flox/flox}* mice develop hematologic disease 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, the MPN in *Mx1-Cre Nf1^{flox/flox}* mice does not spontaneously progress to AML.

Retroviral insertional mutagenesis (RIM) is a powerful strategy for generating hematologic cancers in mice and for identifying genes that contribute to leukemogenesis (22-24). We have made extensive use of MOL4070LTR, a replication competent ecotropic murine leukemia virus that induces myeloid leukemia (25). 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 (26).

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 lead to the production of activated kinases. However, the most common cancer-associated mutations do not lead to the production of proteins with aberrant 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 (27, 28). 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 (29-31).

Attention has therefore turned to developing anti-cancer agents that target effectors of Ras such as Raf, MEK and Akt (**Fig. 1**). An important limitation of this strategy 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 cellular contexts. 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 human NF1-associated tumors and in animal models showing aberrant activation of these pathways support the potential therapeutic benefit of inhibitors of PI3K, mTOR, and MEK (14, 26, 32-35). We have published a number of preclinical trials in accurate mouse models of early stage and advanced hematologic cancers driven by *Nf1* inactivation or oncogenic *Kras* expression (26, 29, 36-38). 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.

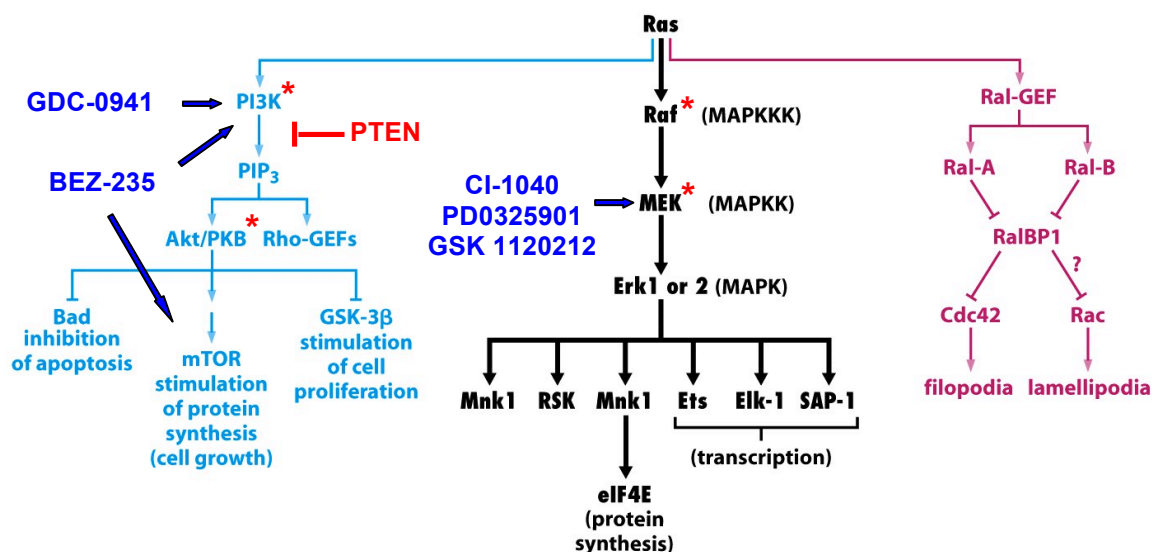


Figure 1. Overview of Major Ras Effector Pathways. Proteins that are altered by oncogenic mutations in human cancer are indicated with red asterisks and PTEN is commonly inactivated in many cancers. Small molecule inhibitors that we have tested in our preclinical models are shown in blue with arrows 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 and pI-pC at day 3 to 5 of life (26). In this screen, *Mx1-Cre, Nf1^{flox/flox}* mice demonstrated a higher incidence of AML as well as reduced latency with myeloblasts visible in blood and marrow and myeloid markers detected by flow cytometry. 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 μ M 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 (26). 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 (26). 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 (39-42). We exploited a shotgun cloning strategy (43) to identify novel retroviral integrations in AMLs 6537R, 6554R1, and 6554R2. This analysis implicated p38 α 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 (26). 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 described above. While the ability to manipulate AML cells *ex vivo* and transplant them provides a novel opportunity to assay the effects of restoring neurofibromin GAP activity on the proliferation and survival of primary *Nf1*-deficient cancer cells *in vivo*, these experiments are technically challenging. Assessing how restoring neurofibromin GAP activity modulates the growth of primary AML cells poses particular difficulties because antibodies to the GAP domain are not currently available, levels of gene expression from viral promoters are unpredictable, and conserved segments of neurofibromin outside of the GAP domain might be essential for biologic activity (44).

Given these practical obstacles, we first focused on developing and validating a lentiviral delivery system for modulating gene expression in primary AML cells generated from MOL4070LTR screens in *Nras* and *Kras* mutant mice (36, 45). Advantages of these models include the small size of the *Nras* and *Kras* genes and the availability of excellent antibodies to both Ras proteins. We first generated shRNA constructs to human *NRAS* and infected a panel of 9 human AML cell lines, including 3 with oncogenic *NRAS* mutations (OCI-AML3, THP-1, and HL60). Two independent hairpins that markedly reduced N-Ras protein levels (**Fig. 2A**) inhibited the growth of all 3 *NRAS* mutant AML cells (**Fig. 2B**). Importantly, this activity was genotype-specific as MV4-11 and other AML cell lines with “driver” mutations in *FLT3*, *CKIT*, or *KRAS* were insensitive to *NRAS* knockdown (**Fig. 2B** and data not shown).

To extend these data to AML cells grown *in vivo*, we designed shRNA constructs to reduce murine *Nras* expression, cloned them into a lentiviral vector containing a red fluorescent protein (mCherry) selectable marker, and infected primary *Nras* mutant AML cells with this construct or with a control Renilla vector. After sorting to isolate mCherry-positive cells, transduced AML cells were injected into irradiated recipient mice. Expressing a potent *Nras* shRNA construct (Nras 30) significantly delayed the onset of AML in transplant recipients compared to mice transplanted with AML cells infected with the control vector (**Fig. 2C**). Importantly, quantitative RT PCR analysis revealed an 80% reduction in *Nras* expression in transplanted AML cells, and showed that the leukemias that ultimately emerged in recipient mice largely restored *Nras* expression (**Fig. 2D**). *Nras* knockdown did not affect the survival of a control retroviral-induced leukemia with wild-type *Nras*.

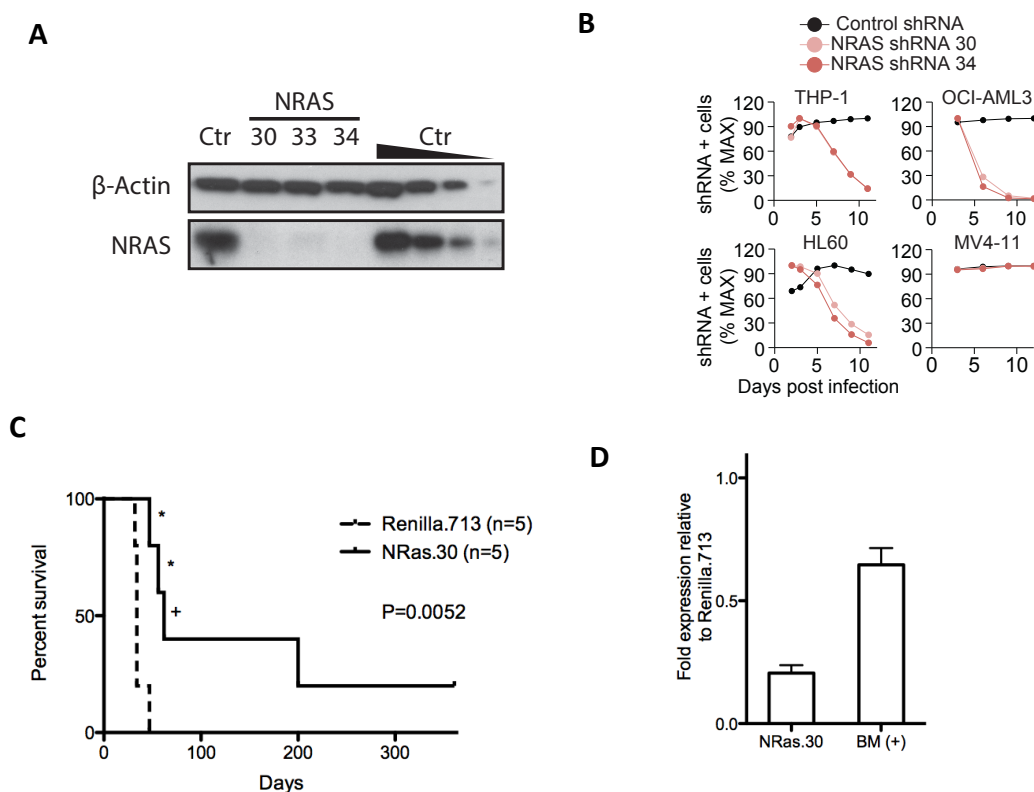


Figure 2. *NRAS/Nras* mutant AML cells are dependent on N-Ras. (A) shRNA constructs 30, 33, and 34 efficiently knockdown human *NRAS* expression in cell lines and (B) inhibit the growth of human AML cell lines with *NRAS* mutations. (C) *Nras* knockdown retards the growth of primary mouse *Nras* mutant AML cells *in vivo* and (D) AML cells that grow out restore *Nras* expression (right) compared to cells that were transplanted (left).

Based on the success of these “proof of concept” studies in *Nras* mutant AML, we are generating lentiviral vectors to express the wild-type neurofibromin GAP domain and a control virus encoding a R1276P amino acid substitution that alters the critical “Arg finger” residue, which is essential for accelerating Ras GTPase activity. Importantly, the substitution was reported in a family with classic NF1 and was shown to markedly impair GAP activity *in vitro* (46). We have modified these constructs to introduce an in frame hemagglutinin (HA) tag that can be used to measure protein expression by Western blotting and for immunoprecipitation experiments to assay GAP activity in transduced cells using a ^{32}P release assay from labeled recombinant Ras-GTP (47, 48).

Our data showing that primary AML cells with oncogenic *Nras* mutations are dependent on aberrant Ras signaling for proliferation and survival *in vivo* suggest that this will also be true of parental *Nf1* mutant AMLs 6537 and 6554. We will test this hypothesis by expressing either the wild-type neurofibromin GAP domain or the R1276P mutant protein in these cells. After validating our lentiviral constructs by assessing GAP activity in cultured cells, we will transduce and transplant primary *Nf1* mutant AMLs 6537 and 6554 and closely monitor the recipients for evidence of disease. We hypothesize that the survival of mice transplanted with AML cells expressing the wild-type GAP domain will be greatly prolonged compared to recipients transplanted with the same AML cells expressing either the control empty vector or a R1276P mutant GAP protein. We will also assess GAP expression and biochemical activity in recipients that develop AML after receiving cells expressing the wild-type GAP domain to ask if they no longer express this protein. We will then perform a similar analysis by transducing and transplanting the three resistant subclones (6537R, 6554R1, and 6554R2). It will be particularly interesting to determine if MEK-resistant subclones differ from the respective parental AMLs with respect to GAP dependence, or if these leukemia cells are no longer require *Nf1* inactivation for survival and proliferation. We will complete these studies in the coming fund year.

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

Our preclinical studies and observations in human patients treated with small molecule inhibitors of signaling molecules have shown that most advanced cancers respond transiently before becoming resistant (26, 49). Emerging data also suggests that combining targeted and conventional chemotherapeutic agents increases efficacy in other cancers (50), and genetically diverse transplantable *Nf1* mutant AMLs provide a robust system for applying this principle to NF1-associated cancers.

The standard treatment for AML in children and adults involves administering high doses of cytarabine in combination with an anthracycline agent such as daunorubicin (51). 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 is 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. We first sought to establish the maximally tolerated dose (MTD) dose of CPX-351 in mice treated with sublethal irradiation and transplanted with primary AML cells. Using a model in which immunodeficient mice were engrafted with a human leukemia cell line, scientists at Celator determined the MTD of CPX-351 at an optimal cytarabine:daunorubicin ratio of 10 mg:4.4 mg (52, 54). The best results were obtained in mice that

were treated on days 1, 3, and 5 (induction phase) and then received 50% of the induction dose on days 21 and 26 (consolidation phase). As expected from studies in human AML, the dose limiting toxicities were weight loss and bone marrow suppression. Based on these data, we tested three induction regimens: the published dose of 10 mg:4.4 mg, as well as doses 20% higher and 20% lower (i.e. 12.5 mg:5.2 mg and 8 mg:3.5 mg). WT mice were first sublethally irradiated with 450 cGy and injected with AML cells with CPX-351 treatment starting 4 days later. All recipient mice treated at each of these initial dose levels died of myelosuppression 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. We recently initiated a MTD study to determine proper dosing and timing for the single agent (CPX-351) and combination (CPX-351 + MEK inhibitor) therapy in WT mice. We also launched a pilot efficacy study in two of our well-characterized AMLs, and are running these studies in parallel. Preliminary data from these ongoing experiments are presented in the following paragraphs.

To evaluate the toxicity of CPX-351 and the conditioning radiation, we sub-lethally irradiated WT mice on day zero and injected them with three doses of CPX-351 at 5 mg:2.2 mg four days after irradiation (days +4, +6, and +8). Mice are being weighed weekly and blood is collected to measure leukocyte and red blood cell counts. After a tolerable combination dose is defined in WT mice, we will perform an MTD study in mice that are transplanted with AML cells on the day they are radiated. We will then enroll 5-7 independent *Nf1* mutant AMLs into cohorts of ~10 mice each for treatment with vehicle control, CPX-351 alone or CPX-351 + PD901 or GSK1120212 (see below).

For the pilot efficacy study, we are testing the combination of CPX-351 and PD901 in mice transplanted with the *Nf1* mutant AML 6537 and with a *Kras*^{G12D} AML called *Kras1*. These leukemias were selected for analysis because they respond dramatically to PD901 treatment, but invariably relapse with clonal evolution. We transplanted whole bone marrow from leukemic donor mice into a sub-lethally irradiated cohort of recipient animals on day 0. The first dose of CPX-351 was given 4 days later for 3 total doses every other day (days +4, +6, and +8 as above). In one cohort, PD901 treatment was started 2 weeks after radiation and transplantation. Recipient mice transplanted with each AML were randomly assigned to receive vehicle control (n=5), CPX-351 (n=5), or CPX-351 + PD901 (n=5). All of the vehicle-treated mice died 10-14 days after transplant from aggressive leukemia (**Fig. 3**). At this writing, mice that received single agent treatment with CPX-351 continue to appear well. While the combination also extended survival compared to the control group, most mice given CPX-351 + PD901 died with evidence of bone marrow failure (**Fig. 3b**). These observations are not unexpected as irradiation, leukemic marrow infiltration, cytotoxic drugs, and PD901 all cause bone marrow suppression.

In summary, we have shown that CPX-351 is well tolerated when administered at the 5 mg:2.2 mg dose level, and also exhibits potent anti-leukemic activity *in vivo* (**Fig. 3**). We are observing recipients of AMLs *Kras1* and 6537 and will isolate blood and bone marrow cells for molecular analysis from mice that develop disease. We are excited about these data, which indicate that we can administer “standard of care” AML treatment in this model. We will soon launch new trials treating at this dose of CPX-351 while waiting 28 days before starting PD901. If toxicity remains unacceptable, we will treat with PD901 4 days a week, which is efficacious when combined with the PI3 kinase inhibition GDC-0941 in a model of T lineage acute lymphoblastic leukemia (data not shown).

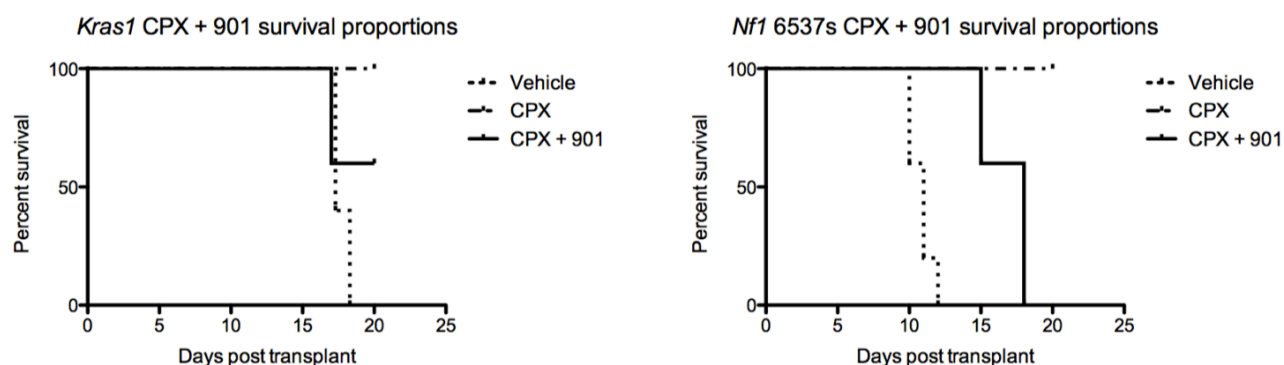


Figure 3. Treatment of *Kras*^{G12D} and *Nf1*^{-/-} AMLs with CPX-351 (CPX) alone and in combination with PD0325901 (901). Kaplan-Meier analysis demonstrates improved survival in mice assigned to treatment with CPX (dashed line) compared to recipients transplanted with the same leukemias that received control vehicle (dotted line). Mice assigned to combination therapy with CPX and 901 suffered early mortality secondary to bone marrow suppression.

Preclinical evaluation of MEK inhibitor GSK1120212 in *Nf1* Mutant Mice with Myeloid Disease

Retinal vein occlusion and neuropathies were uncommon but serious complications observed in patients enrolled in early phase trials of PD901 and similar MEK inhibitors (55). GSK1120212 (trametinib; Glaxo-Smith-Kline) is a potent and selective allosteric MEK inhibitor (56) that is considered “best in class” and was recently approved for treatment in melanoma by the FDA. In addition to showing a superior side effect profile than PD901 in human patients (less myelosuppression), GSK1120212 (GSK ‘212) is currently being evaluated in at least 13 phase I, II, and III clinical trials of adults with cancer, and recently received FDA-approval for treatment of BRAF_{V600E} mutant melanoma.

We are committed to investigating drugs and drug combinations that can be translated into clinical trials of *Nf1*-associated cancers, and therefore executed a material transfer agreement (MTA) to test GSK ‘212 in mouse models of *Nf1* mutant MPN and AML. We have assessed GSK ‘212 in parallel with PD901 to compare the pharmacodynamic properties, safety profiles, and preclinical efficacy of these MEK inhibitors. We have begun trials to define a maximum tolerated dose (MTD), and report here data from pilot trials in *Mx1-Cre; Nf1^{flox/flox}* mice with MPN as well as a more aggressive *Mx1-Cre; Kras^{G12D}* murine model of MPN. Our ultimate goal is to substitute GSK ‘212 in place of PD901 in combination AML studies.

We launched an efficacy trial of GSK ‘212 in *Mx1-Cre; Nf1^{flox/flox}* and *Mx1-Cre; Kras^{G12D}* mice with MPN (57, 58) to ask if the enhanced pharmacologic properties of GSK ‘212 is associated with better preclinical efficacy. In these studies, we administered GSK ‘212 at a dose suggested by the GSK scientific team (2 mg/kg/day), enrolling 8-10 mice per arm in the *Nf1* cohort and 3 mice per arm in the *Kras* mutant cohort. After 21 days of treatment, *Nf1* mutant mice and WT mice treated with GSK ‘212 developed a progressive erythematous, pruritic rash on their snouts and chest. These mice also experienced weight loss, likely due to the rash pain associated with eating. The dose was lowered to 1 mg/kg/day but the rash persisted and the drug was discontinued in *Nf1* mice. The *Kras* mice are younger at the start of trial (2 months vs. 6 months in the *Nf1* group) and did not develop a rash as quickly as the *Nf1* mice. After 21 days, the dose was decreased to 1mg/kg due to the same skin toxicity seen in the *Nf1* mice. By the end of the trial (10 weeks), *Kras* mutant mice treated with GSK ‘212 also exhibited progressive rash and weight loss. These mice also showed dramatic improvement of hematologic parameters and prolongation of survival (**Fig. 4A-D**). Interestingly, *Kras* mice treated

with GSK '212 initially showed improvement of their anemia, but began showing a decrease in hemoglobin concentration toward the end of the trial (**Fig. 4B**).

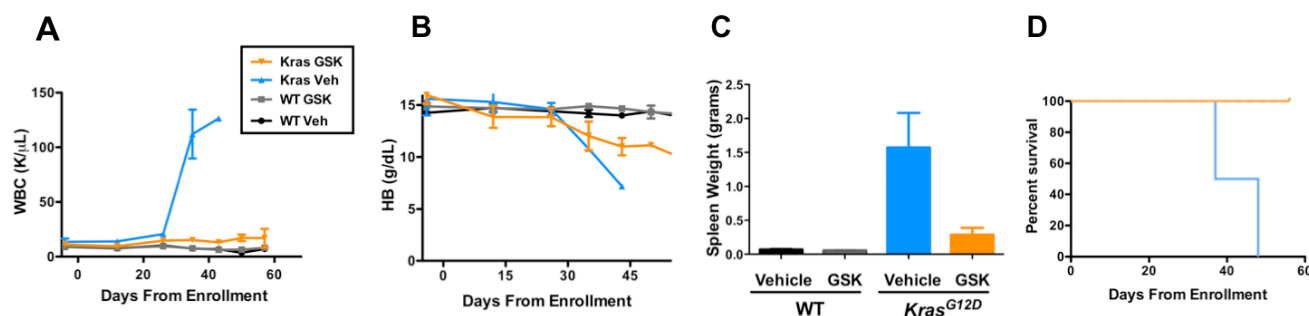


Figure 4. Response of *Mx1-Cre, Kras^{G12D}* Mice with MPN to GSK 1120212 (GSK). In all panels, *Mx1-Cre, Kras^{G12D}* Mice with MPN mice treated with control vehicle are shown in blue; *Kras^{G12D}* mice treated with GSK '212 are in orange; WT mice given the vehicle are in black; and WT mice treated with GSK '212 are in gray. **A.** Blood leukocyte counts. **B.** Hemoglobin concentration. **C.** Spleen weight at the end of treatment. **D.** Survival through the end of treatment.

GSK'212 is a potent MEK inhibitor, and our data suggest that the 1-2 mg/kg/day is efficacious, but also substantially more toxic than PD901 at 5 mg/kg/day. We are currently performing a MTD trial in WT mice investigating GSK '212 doses of 0.5 mg/kg and 1 mg/kg. To date, mice have been treated on this trial for almost a month and appear well with no skin toxicity or weight loss. We recently completed a pharmacodynamic experiment in which we assessed pERK inhibition in bone marrow cells isolated from WT mice given 0.5 or 1.0 mg/kg/day of GSK '212. An analysis of these data revealed comparable target inhibition in mice given 0.5 mg/kg/day as we previously observed at the 5 mg/kg dose of PD901 (**Fig. 5**) (37, 38). We are currently expanding our *Mx1-Cre; Nf1^{flox/flox}* and *Mx1-Cre; Kras^{G12D}* colonies and will initiate a new 0.5 mg/kg/day efficacy trial of GSK'212 in both MPN models in the next few weeks. Data from these studies will inform our subsequent work in mice with AML as we hope to replace PD901 with GSK'212 to facilitate translation into human NF1 patients with JMML, AML, and other cancers.

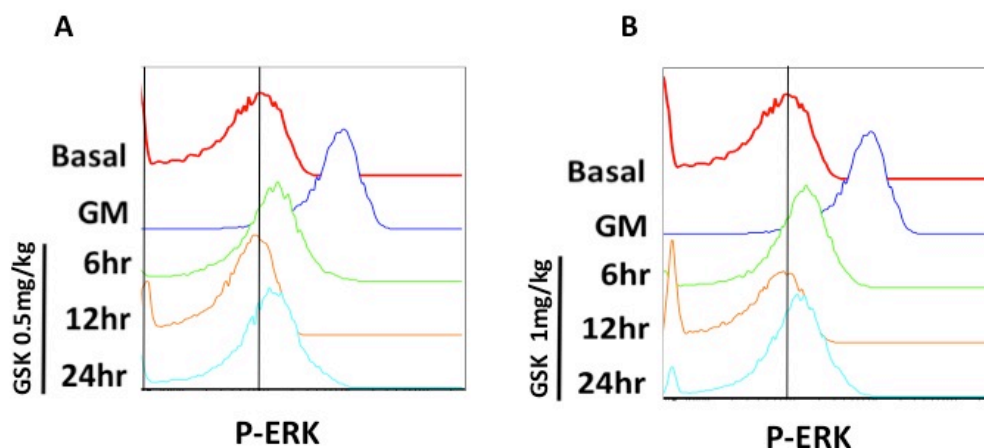


Figure 5. GSK1120212 inhibits MEK in vivo. Mice were treated with 0.5mg/kg/day (**A**) or 1mg/kg/day (**B**) of GSK1120212 (GSK) for 5 days, then euthanized 6, 12, or 24 hours after the final dose. Phosphorylated ERK (pERK) levels were measured by flow cytometry in *Mac1⁺ Gr1⁺* bone marrow cells after stimulation with a saturating dose of GM-CSF (10ng/mL). Both doses achieve comparable target inhibition of p-ERK, with complete shutdown at 12 hours and slight return of signaling at 24 hours.

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 GSK'212), 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 the CPX-351 data shown in Figure 3 and our expectation that we will develop combination regimens that will bypass the cumulative toxicity observed to date in mice given CPX-351 + PD901.

KEY RESEARCH ACCOMPLISHMENTS

- (a) We developed a lentiviral transduction/transplantation system for assaying the effects of modulating gene expression on the growth of AML cells *in vivo*, and are generating constructs to assess how restoring neurofibromin GAP activity modulates the growth of primary *Nf1* mutant AMLs. These studies will compare the effects of expressing the GAP domain in AML cells that are sensitive to PD901 and in resistant subclones of these primary leukemias.
- (b) We established a tolerable dose of CPX-351 in our background strain. Compared to vehicle-treated controls, treatment with CPX-351 markedly prolongs survival in recipient mice transplanted with *Nf1* and *Kras* mutant AMLs.
- (c) We found that a daily dose of 2 mg/kg/day of the MEK inhibitor GSK'212 causes excessive toxicity in WT mice and in mice with MPN, and are conducting MTD studies at two lower dose levels (0.5 and 1 mg/kg). We have established that the 0.5 and 1 mg/kg doses result in excellent pharmacodynamic target inhibition.
- (d) We showed that 1mg/kg/day of GSK 1120212 induces a rapid and durable decrease in leukocytes, improvement of anemia, reduction in spleen size and prolonged survival in *Kras* mutant mice with MPN.

REPORTABLE OUTCOMES

(a) Research Articles & Reviews

None to date.

(b) Abstracts

None to date.

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

Leukemia & Lymphoma Society Special Fellow Award (awarded to Tiffany Chang, MD)

(d) Employment and research opportunities

This award has provided salary support for technical personnel in the lab

CONCLUSIONS

As summarized in the “Key Research Accomplishments” section above, we have made progress toward executing the aims 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*. These vectors now allow us to assess the effects of restoring neurofibromin GAP activity in primary *Nf1* mutant AML cells. We have advanced the goals of Aim 2 by establishing a tolerable dose of CPX-351 in our background strain. Based on exciting preliminary data showing that treatment with CPX-351 markedly prolongs survival in recipient mice transplanted with *Nf1* and *Kras* mutant AMLs, we expect to isolate mutant subclones for molecular analysis as proposed in Aim 3. In work not proposed in our original Aims, but that is integral to the long term goal of translating data obtained from preclinical trials in genetically accurate mouse models of NF1-associated cancers to human patients, we have begun studies to investigate GSK1120212, a novel MEK inhibitor that was recently approved by the FDA. 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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