AWARD NUMBER: W81XWH-14-1-0324

TITLE: GrB-TWEAK: A Potential Novel Biologic for NSCLC Therapy

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REPORT DATE: July 2017

TYPE OF REPORT: Addendum to Final

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

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED			
	Addendum to Final	1Sept 2016 - 30 Apr 2017			
4. IIILE AND SUBTILE		5a. CONTRACT NUMBER			
GrB-TWEAK: A Potential Novel Biologic for NSCLC					
		5b. GRANT NUMBER			
		VV81XVVH-14-1-0324			
		5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S)		5d. PROJECT NUMBER			
Jeffrey A. Winkles, Ph.D.					
		5e. TASK NUMBER			
		5f. WORK UNIT NUMBER			
E-Mail: iwinkles@som umarvland	edu				
7. PERFORMING ORGANIZATION NAM	E(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT			
		NUMBER			
University of Maryland, Baltimore					
Baltimore, MD 21201					
,					
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)			
LLC Army Medical Desserve and	Material Command				
U.S. Army Medical Research and Materiel Command					
Fort Detrick, Maryland 21/02-50	12	11. SPONSOR/MONITOR'S REPORT			
		NUMBER(S)			
12. DISTRIBUTION / AVAILABILITY STA	ATEMENT				
Approved for Public Release; Dis	tribution Unlimited				
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
My laboratory studies the cytokine named TWEAK and its cell surface receptor named En14 and their role in					
cancer biology. We reported previously that En14 is expressed at low levels in normal lung tissue but biobly					
expressed in many nen small call lung concers (NICCI Co). Additionally, in collaboration with Dr. Decembly in					
expressed in many non-small cell lung cancers (INSULUS). Additionally, in collaboration with Dr. Rosenblum's					
research group we have successfully developed several Fn14-targeted fusion proteins that exhibit cytotoxic activity					
on cancer cells in vitro and in vivo. In this Lung Cancer Idea Award application we proposed to test the effects of					
these fusion proteins, and in particular the GrB-Fc-IT4 construct, on NSCLC cell growth in vitro (Aim 1) and in vivo					
(Aim 2). During year 1, we were able to demonstrate that two different En14-targeted proteins that use granzyme B					
(GrB) as the cell killing agent (TWEAK-GrB, GrB-Ec-IT4) exhibit pro-apontotic activity when added to numerous					
En14 positive NSCLC cell lines. During year 2, we conducted additional work to inform the design of the in vivo					
atudice. During our no cost extension, we worked with Dr. Denors grown to test whether the OrD Dr. 174 construct					
studies. During our no-cost extension, we worked with Dr. Berens group to test whether the GrB-Fc-IT4 construct					
could inhibit lung cancer PDX growth in vivo. This data is described in this FINAL REPORT ADDENDUM.					
15. SUBJECT TERMS					
TWEAK, En14, targeted therapy, granzyme B, lung cancer					
TWEAR, THIT, LAIGUEU UICIAPY, GIANZYING D, IUNG CANCEI					

16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	5	19b. TELEPHONE NUMBER (include area code)
Unclassified	Unclassified	Unclassified			

PI: Jeffrey A. Winkles, Ph.D. DoD Award W81XWH-14-1-0324

FINAL REPORT ADDENDUM

1. Accomplishments:

During this no-cost extension period we were able to test whether our therapeutic construct GrB-Fc-IT4 could inhibit NSCLC tumor growth *in vivo* using the NSCLC patient-derived Fn14-positive cell line M2010-1005. The M2010-1005 line was established from a NSCLC patient with a tumor harboring the EGFR^{del747-752}-activating mutation and, as expected (Whitsett et al., <u>American Journal of Pathology</u> 181:111-120), Fn14 expression is robust in this line (Fig. 1A). M2010-1005 maintains histological (Fig. 1B) and genetic (unpublished data) characteristics of the original patient tumor during *in vivo* passaging.



Figure 1. Properties of the patient-derived NSCLC explant M2010-1005. A. Fn14 mRNA expression levels in two patient-derived NSCLC explants was assessed by real-time PCR. Relative fold change calculated by $\Delta\Delta$ Ct method with histone as a reference gene is shown. B. Histological characteristics of the P2010-1005 patient tumor and the M2010-1005 explant at *in vivo* passage number 3 (H & E staining). Genetic characteristics of the P2010-1005 tumor, as analyzed by array CGH, are also maintained during *in vivo* passaging (data not shown).

Animal experiments to test GrB-Fc-IT4 antitumor efficacy were conducted via subcontract with Dr. Michael Berens at TGen (Phoenix, AZ) following approval by the Institutional Animal Care and Use Committee guidelines of St. Joseph's Hospital and Medical Center. Studies were carried out in nu/nu athymic mice (Jackson Labs) maintained under pathogen-free conditions and a 12-hour light/12-hour dark cycle. Fresh M2010-1005 tumor tissue originally resected from a patient at the time of surgery, with informed, written patient consent, was implanted subcutaneously (s.c.) into the flanks of 6-week-old nu/nu mice. Xenografts were allowed to grow to approximately 200 mm³. Animals were grouped randomly (6 mice per group) and treated with vehicle (sterile PBS) or GrB-Fc-IT4 (20 mg/kg) every other day over a ten day period for a total dose of 100 mg/kg via tail vein injection. Tumor size was measured 2-3 times per week by caliper measurements and volumes were calculated using the following formula: V = (a X b2) / 2, where 'a' is the largest dimension and 'b' the smallest. Tumor growth in GrB-Fc-IT4-treated animals was compared to vehicle-treated mice. We found that GrB-Fc-IT4 was able to inhibit tumor growth in this lung cancer PDX model (Fig. 2).



Figure 2. Antitumor efficacy of GrB-Fc-IT4 protein in M2010-1005 NSCLC PDX model. M2010-1005 tumor tissue was injected subcutaneously into nude mice and tumors were allowed to grow to ~200 mm³ in volume. Groups (n=6) were injected (iv, tail vein) with either saline (control) or GrB-Fc-IT4 at 20 mg/kg every other day over a ten day period for a total dose of 100 mg/kg. Tumor size was assessed by direct caliper measurements and tumor volumes calculated. Efficacy data is plotted as mean tumor volume +/- SEM.

In summary, in this Lung Cancer Idea Award application we proposed to test the effects of new Fn14-targated fusion proteins, and in particular the GrB-Fc-IT4 construct, on NSCLC cell growth in vitro (Aim 1) and in vivo (Aim 2). We have accomplished these goals and more research is in progress. Notably, one of my collaborators on this project, Dr. Michael Rosenblum, has received financial support to begin work that we hope will move the GrB-Fc-IT4 therapeutic agent into a Phase 1 clinical trial (see abstract mentioned below).

2. Products (Reportable Outcomes from No-Cost Extension):

We have submitted an abstract describing the *in vivo* results for presentation at a scientific meeting. The meeting information and abstract is on the next page.

AACR Conference on Tumor Immunology and Immunotherapy, October 1-4, 2017; Boston MA

Targeted Granzyme B Immunotherapy: A Novel Approach Delivering GrB to Fn14-Positive Solid Tumors

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All immune effector cells including engineered T-cells and other immunotherapeutic approaches rely on the delivery of the serine protease granzyme B (GrB) to the target cells resulting in a potent cytotoxic (apoptotic) effect that has been well characterized. We have developed a series of novel, completely human constructs for targeted, tumor antigen-mediated delivery of GrB without the need for effector cells. These bivalent constructs can be targeted to cells by incorporating scFv proteins that bind cellsurface antigens. A series of GrB-based constructs are included in our Targeted GrB Immunotherapy (TGI) platform. Our initial focus for further development is GrB-Fc-IT4 (MRT-101), a dimeric, bivalent construct with a high molecular weight (160 kDa) that binds the TWEAK receptor fibroblast growth factor-inducible protein 14 (Fn14). This receptor is highly over-expressed in many solid tumor types. The construct was expressed in HEK293E suspension cells, harvested under serum-free conditions and purified to homogeneity. The specific activity of the GrB moiety, as assessed by cleavage of a synthetic chromogenic GrB substrate, was comparable to natural GrB. Against a panel of >40 human cancer cell lines expressing Fn14, MRT-101 showed high affinity and selective cytotoxicity in the nanomolar range (IC₅₀ ranged from 4 to 284 nM) and was 2-100X more potent than free GrB. Mechanistic studies demonstrated that MRT-101 activated caspase cascades and cytochrome C related pro-apoptotic mechanisms consistent with the known intracellular functions of GrB. Pharmacokinetic studies in mice revealed that the MRT-101 fusion protein exhibited a bi-exponential clearance from plasma with a rapid initial clearance (t $_{\frac{1}{2}\alpha}$ = 0.36 hours) followed by a prolonged terminal-phase plasma half-life (t $_{\frac{1}{2}\beta}$ = 35 hours). Toxicity studies in mice demonstrated that the MTD exceeds a cumulative dose of 100 mg/kg. Athymic nude mice bearing MDA-MB-231 orthotopic breast tumor xenografts were intravenously treated with saline or MRT-101 construct (Qod X 5). On average, tumors from saline-treated mice grew ~10-fold over 40 days. In contrast, tumors from mice treated with MRT-101 showed no growth over this period. Moreover, 3/5 mice treated with MRT-101 had complete tumor regression lasting beyond day 80 (end of the study). A second study in NSG-SCID mice bearing well-developed MDA-MB-231 orthotopic tumors showed significant tumor growth inhibition by MRT-101 in both intravenous and intraperitoneal treatment groups. Mice bearing subcutaneous lung adenocarcinoma PDX tumors treated with MRT-101 also demonstrated >50% tumor suppression vs saline controls. Overall, treatment with MRT-101 was well-tolerated (no loss of body weight) and demonstrated significant anti-tumor efficacy against multiple tumor types. These studies represent the characterization of a new class of targeted immunotherapeutics containing a synthetic GrB payload identical to that of immune effector cells. The MRT-101 fusion construct is currently undergoing IND-enabling studies and GMP manufacturing in advance of projected Phase 1 clinical trials. Research conducted, in part, by the Clayton Foundation for Research.