

AWARD NUMBER: W81XWH-13-1-0227

TITLE: Deficient BIM Expression as a Mechanism of Intrinsic and Acquired Resistance to Targeted Therapies in EGFR-Mutant and ALK-Positive Lung Cancers

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REPORT DATE: August 2014

TYPE OF REPORT: Annual

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

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE August 2014		2. REPORT TYPE Annual		3. DATES COVERED 1Aug2013-31July2014	
4. TITLE AND SUBTITLE Deficient BIM Expression as a Mechanism of Intrinsic and Acquired Resistance to Targeted Therapies in EGFR-Mutant and ALK-Positive Lung Cancers				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0227	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jeffrey Engelman MD PhD and Lecia Sequist MD E-Mail: jengelman@partners.org ; lsequist@partners.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Massachusetts General Hospital Boston, MA 02114				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 The project has had very good progress over the past year. There have been a few developments that have accelerated discovery. We have been able to develop cell lines directly from patient biopsies after the development of resistance in the clinic. We had not had this capability when we applied for this award. We can now use these clinically relevant models to assess the expression of BIM and induction of apoptosis. We are also able to test new therapeutic strategies to overcome the low apoptotic rates. These studies led to the identification of the combination of ABT-263 and irreversible EGFR inhibitors to treat T790M containing EGFR mutant cancers as planned in Aim 3 of this award. The success of these studies has led to a clinical trial sponsored by CTEP combining AZD9291 and ABT263. This should enter the clinic in 2015. We have also worked hard to validate a robust, quantitative IHC method for measuring BIM. This will inform the value of this biomarker to determine which patients will benefit most from this combination.					
15. SUBJECT TERMS BIM, apoptosis, targeted therapy, BCL-XL, kinase, EGFR, ALK, lung cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In lung cancers that have oncogene-addiction to a specific kinase, inhibition of that kinase often leads to cell growth arrest and apoptosis. For example, *EGFR* mutant and *EML4-ALK* lung cancers have been proven highly sensitive to the corresponding specific tyrosine kinase inhibitors (TKIs). Although cancers with these genetic abnormalities often respond to the appropriate targeted therapy, there is marked heterogeneity in degree of clinical benefit. We hypothesized that that some cancers are poised to undergo apoptosis following treatment, whereas others are not; and expression of the critical pro-apoptotic protein BIM in pre-treatment biopsies may distinguish patients who have impressive, durable responses from those who have weak, transient responses. We aimed to assess *EGFR* mutant and *EML4-ALK* lung cancer specimens to determine if low basal BIM expression predicts a poorer clinical outcome to TKIs. We also aimed to leverage our robust repeat biopsy program that routinely biopsies patients upon the development of resistance to determine if BIM levels are depressed in patients' specimens following acquired resistance to TKIs. These studies will be complemented by interrogation of patient-derived cell lines with acquired resistance to TKIs to determine whether apoptosis and Bcl-2 family proteins, with special emphasis on BIM, are altered in resistant cancers, thereby mitigating responsiveness to subsequent therapies. For cancers with low endogenous levels of BIM, we aimed to determine if epigenetic silencing of the *BIM* locus is responsible for its repressed expression, and we to systematically examine therapeutic strategies to either de-repress endogenous BIM expression or maximize the amount of "free" BIM by targeting BIM inhibitors in the cell (BH3 mimetics) to sensitize cancers to targeted therapies.

2.KEYWORDS: Provide a brief list of keywords (limit to 20 words).

BIM, apoptosis, targeted therapy, BCL-XL, kinase, EGFR, ALK, lung cancer

3.OVERALL PROJECT SUMMARY: The project has had very good progress over the past year. There have been a few developments that have accelerated discovery. Since obtaining this award we have accumulated baseline (pre-treatment) material from 129 EGFR mutant lung cancer patients and 92 biopsies on ALK-translocated patients. This has been accomplished through our repeat biopsy program. Therefore, we have already exceeded the number of samples proposed for the entire award. We have been able to develop cell lines directly from patient biopsies after the development of resistance in the clinic (Crystal et al, Science, 2014). We had not had this capability when we applied for this award. We can now use these clinically relevant models to assess the expression of BIM and induction of apoptosis.

We are also able to test new therapeutic strategies to overcome the low apoptotic rates. These studies led to the identification of the combination of ABT-263 and irreversible EGFR inhibitors to treat T790M containing EGFR mutant cancers as planned in Aim 3 of this award. The success of these studies has led to a clinical trial sponsored by CTEP combining AZD9291 and ABT263. This should enter the clinic in 2015.

We have also worked hard to validate a robust, quantitative IHC method for measuring BIM. This will inform the value of this biomarker to determine which patients will benefit most from this combination.

Our research has expanded beyond just assessing BIM but is also focusing on the role of a diminished apoptotic response as clones develop resistance to targeted therapies. Although BIM is one such mechanism, it is not the only one. We are now also trying to identify the other mechanisms as well.

Aim 1: Validate BIM as a biomarker that predicts outcome in patients treated with EGFR and ALK inhibitors.

Task 1:

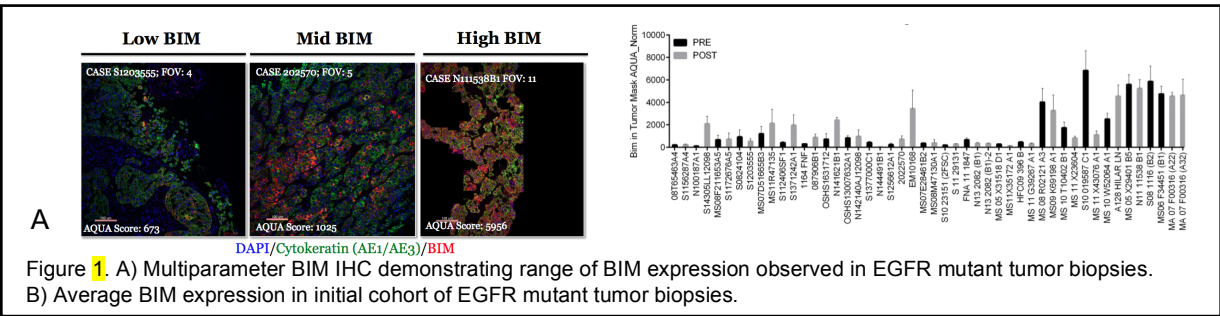
Current objectives: obtain biopsies from 100 *EGFR* mutant lung cancer patients and 60 *ALK* translocated lung cancer specimens prior to TKI treatment.

Summary of Results, Progress and Accomplishments: Since obtaining this award we have accumulated baseline (pre-treatment) material from > 150 *EGFR* mutant lung cancer patients and 92 biopsies on *ALK*-translocated patients (See Tables Below). This has been accomplished through our repeat biopsy program. Therefore, we have already exceeded the number of samples proposed for the entire award.

Subtasks.

1A. Current objectives: Perform BIM IHC and RNA ISH on approximately 100 pre-treatment *EGFR* mutant lung cancer specimens. These slides will be processed from paraffin-embedded tissue.

Summary of Results, Progress and Accomplishments: We have worked closely with Dr. David Rimm at Yale University to develop a robust, quantitative IHC for the detection of BIM. The development of this assay has been developed and validated using a series of control specimens. A set of 25 of paired *EGFR*



mutant samples have been sent to Dr. Rimm for analysis. Initial results are shown in Figure 1. This assay should be a very powerful method to determine if low BIM predicts for worse outcome and if BIM is lost upon the development of resistance to targeted therapies.

1B. Current objectives: Perform BIM IHC and RNA ISH on approximately 60 pre-treatment *ALK* translocated lung cancer specimens. These slides will be processed from paraffin-embedded tissue.

Summary of Results, Progress and Accomplishments: We have already collected more than 90 *ALK* lung cancer specimens. We are evaluating the results from the initial IHC from the *EGFR* samples noted above before these are sent to Dr. Rimm for analysis.

1C. Current objectives: Prospectively analyze progression free survival (PFS) in relationship with BIM expression, as determined by IHC and RNA ISH.

Summary of Results, Progress and Accomplishments: There is a delay in being able to analyze PFS as the median time to reach this endpoint for lung cancer patients on targeted therapies is 10 months. Patients are being followed for this outcome so that analysis by BIM expression can be analyzed. As mentioned above, we are now determining the baseline BIM levels using quantitative IHC. These results will be used to determine if BIM levels predict for clinical benefit from *EGFR* and *ALK* inhibitors.

Aim 2: Determine if BIM expression is lost in cancers that develop resistance to targeted therapies.

Task 2:

Current Objectives: Interrogate approximately 50 *EGFR* mutant lung cancers pre- and post-treated matched specimens that have acquired resistance to TKI and approximately 30 *ALK* translocated lung cancer pre- and post-treated matched specimens that have acquired resistance to TKI.

Summary of Results, Progress and Accomplishments: Since obtaining this award we have accumulated matched pre- and post-treatment paired tumor material from 91 *EGFR* mutant lung cancer patients and 59 *ALK*-translocated patients (see Table below). We have also been able to develop cell lines from some of these biopsies. We have determined that BIM expression is lost in a subset of the resistant cancers that are insensitive to subsequent *EGFR* TKIs.

Pre- <i>EGFR</i> TKI Specimens	Post- <i>EGFR</i> TKI Specimens	Paired Pre-/Post- <i>EGFR</i> TKI Specimens
125	122	91

Pre- <i>ALK</i> TKI Specimens	Post- <i>ALK</i> TKI Specimens	Paired Pre-/Post- <i>ALK</i> TKI Specimens
92	88	59

Subtasks.

2A. Current objectives: Make *EGFR* mutant cell lines resistant to *EGFR* TKI gefitinib (cell-lines commercially available from ATCC or acquired from the Center for Molecular Therapeutics at Mass General Hospital Cancer Center).

Summary of Results, Progress and Accomplishments: We have generated resistant cell lines from five commercially available *EGFR* mutant NSCLC cell lines (PC9, H3255, HCC827, H1975, H4006) as well as cell lines derived from patient biopsies. We have taken the T790M+ patient derived models and generated in vitro resistance to 3rd generation *EGFR* TKI in these models as well (please see Table below).

Cell Line	Method	Drug	Number of models	Resistance Mechanism(s)
PC9	Increasing Dose	Gefitinib	3	T790M(2), Sustained ERK act. (1)
HCC827	Increasing Dose	Gefitinib	1	MET Amplification
H3255	Increasing Dose	Gefitinib	1	T790M
H1975	Increasing Dose	Dacomitinib	2	EMT
MGH119	Increasing Dose	Gefitinib	1	T790M
MGH119-GR*	Increasing Dose	WZ4002	5	Sustained Akt act.
MGH119	High Initial Dose	WZ4002	2	Sustained ERK and Akt act.
MGH121	Increasing Dose	WZ4002	5	EGFR C797S (1), Sustained ERK (2), ??? (2)
MGH134	Increasing Dose	WZ4002	4	Unknown
MGH134	High Initial Dose	WZ4002	In process	Unknown
MGH141	Increasing Dose	WZ4002	3	Unknown
MGH164	Increasing Dose	WZ4002	In process	Unknown

* MGH119-GR is the gefitinib resistant (T790M+) model derived from MGH119 pt

We have also made cell lines directly from patient biopsies after acquisition of resistance in the clinic. This approach was featured in Science magazine (Crystal et al, *Science*, 2014). To date, we have made over 35 such models and have 90 additional ones in progress (see Table below).

Drug of Resistance	Naïve	Erlotinib	Gefitinib	Afatinib	Dacomitinib	Clovis	AZD9291	Total
Finished	2	24	0	4	3	4	0	37
In process	3	58	1	10	0	21	0	93

2B. Current objectives: Make *ALK* translocated cell lines resistant to the ALK TKI crizotinib (cell lines commercially available from ATCC or acquired from the Center for Molecular Therapeutics at Mass General Hospital Cancer Center, all readily identifiable).

Summary of Results, Progress and Accomplishments: We have generated resistant cell lines from two commercially available ALK positive NSCLC cell lines (H2228 and 3122) as well as MGH006, a cell line we derive from a patient. Please see Table below.

Cell Line	Method	Drug	Number of models	Resistance Mechanism(s)
H3122	Increasing Dose	crizotinib	3	L1196M (1), 1151Tins (1), EGFR and HER3 act. (1)
MGH006	Increasing Dose	crizotinib	2	SRC activation (1), Unknown (1)
H2228	Increasing Dose	crizotinib	2	SRC activation (1), Unknown (1)
H3122	Increasing Dose	Ceritinib	4	TMEM87B-MERTK fusion (1), EGFR act. (1) Sustained ERK act. (2)
H3122	mice xenografts	Ceritinib	5	Sustained ERK and Akt act. (5)
MGH006	mice xenografts	PF3922	3	Sustained ERK and Akt act. (2), Sustained mTORC1 act. (1)
H3122	Increasing Dose	PF3922	3	Sustained mTORC1 act. (3)
H3122	mice brain metastasis	PF3922	2	Sustained ERK and Akt act. (1), Sustained mTORC1 act. (1)

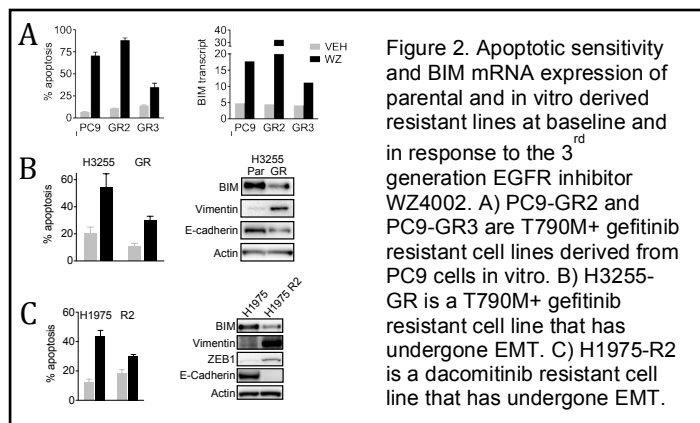
We have also made cell lines directly from biopsies of ALK positive cancers with acquired resistance crizotinib and several second-generation ALK inhibitors.

Drug of Resistance	Naïve	Crizotinib	LDK378	Chugai	Alectinib	AP26113	PF3922	Total
Finished	5	11	9	1	0	0	0	26
In process	0	11	8	0	2	1	3	25

2C. Current objectives: Determine if BIM levels are depressed (or BIM has reduced functionality) in resistant cell lines compared to parental cell lines by Western blot analysis and immunoprecipitation

analysis, and whether resistant lines have a depressed apoptotic response to second-line targeted therapies by FACS analysis.

Summary of Results, Progress and Accomplishments: We have analyzed apoptotic response of each of the resistant lines to irreversible EGFR inhibitors and observed that several of the lines have decreased apoptotic response. We have analyzed BIM protein and RNA expression in each of these cell lines and observe that a subset of resistant cell lines with reduced apoptotic response have decreased BIM levels either at baseline or have reduced induction of BIM after drug treatment. This is especially true in those cancers that have undergone epithelial to mesenchymal transitions (Figure 2). However, some resistant cancers with depressed apoptotic responses do not have suppressed levels of BIM, demonstrating that BIM alone does not explain the decreased apoptotic response in all cases.



2D. Current objectives Make approximately 10 patient-derived resistant cell line models by establishing cell lines from patients with treatment-naïve *EGFR* mutant lung cancers, and exposing them in vitro in increasing concentrations of EGFR TKI

Summary of Results, Progress and Accomplishments: We have generated

one resistant cell line model from the patient-derived cell line MGH119, which was derived from a treatment-naïve EGFR mutant NSCLC. Additionally we recently developed the technical expertise to generate resistant cell lines from patients at the time of clinical disease progression. This has transformed our research because we can now study resistant cell lines from biopsies rather than cultivating resistance in vitro using established cell lines. Over twenty of these lines have been available for characterization and over 75 cell lines are currently under development (see Table above).

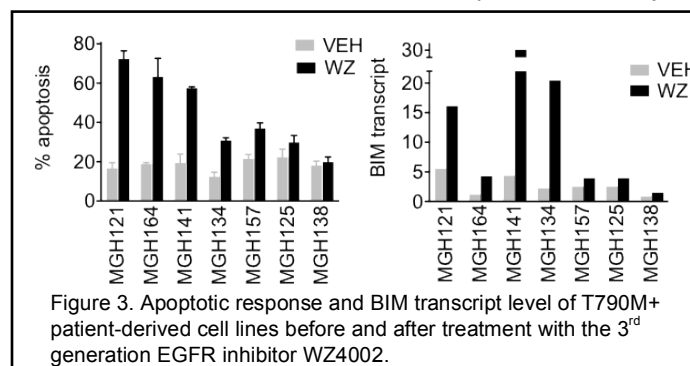
2E. Current objectives: Make approximately 10 patient-derived resistant cell line models by establishing cell lines from patients with treatment-naïve *ALK* translocated lung cancers, and exposing them *in vitro* to increasing concentrations of ALK TKI

Summary of Results, Progress and Accomplishments: As discussed above, we have been able to develop cell lines directly from patients with ALK-positive lung cancers. We have developed > 20 such samples and we have several more in development (see Table above). These are both form ALK TKI naïve and resistant cancers. The resistant samples are being examined in the studies listed below. In particular, we are determining if they have a defect in the apoptotic response to ALK TKIs due to suppressed levels of BIM.

2F. Current objectives: Determine if BIM levels are depressed (or BIM has reduced functionality) in patient-derived resistant cell lines compared to treatment-naïve patient-derived cell lines by Western blot analysis and immunoprecipitation analysis.

Summary of Results, Progress and Accomplishments: Please see descriptions above (repeated again here). We have analyzed apoptotic response of each of the resistant lines to irreversible EGFR inhibitors and observed that several of the lines have decreased apoptotic response (Figure 3). We have analyzed BIM protein and RNA expression in each of these cell lines and observe that a subset of resistant cell lines with reduced apoptotic response have decreased BIM levels either at baseline or have reduced induction of BIM after drug treatment. This is especially true in those cancers that have undergone epithelial to mesenchymal transitions. However, some resistant cancers with depressed apoptotic responses do not have suppressed levels of BIM, demonstrating that BIM alone does not explain the decreased apoptotic response in all cases.

2G. Current objectives: Determine if any of these acquired resistant cell line models and patient-derived resistant models have common, already identified major resistant mechanisms (such as EGFR T790M)



and whether they have a depressed apoptotic response to second-line targeted therapies.

Summary of Results, Progress and Accomplishments: We have performed several studies addressing this question. To our surprise, we have learned that several resistant cancers with other bona fide mechanisms of resistance also develop an impaired apoptotic response to therapies

that overcome the primary resistance mechanism. Several of the acquired resistant models have T790M (PC9, H3255, MGH119) and HCC827 has MET amplification. Of 2 PC9 resistant lines that have T790M, one has depressed apoptotic response to irreversible EGFR inhibitors and the other does not. Both H3255 and MGH119 gefitinib resistant models have depressed apoptotic response. Of the cell lines derived from resistant patients that have been characterized, 7 have T790M. About half of these have decreased apoptotic response to irreversible EGFR inhibitors.

2H. Current objectives: In acquired resistant models that do not have common major resistant mechanisms, we will determine if low BIM is a primary major resistant mechanism, by introduction of pTREX BIM into cells followed by treatment with the relevant TKI. Similarly, with resistant models identified with other major resistant mechanisms, pTREX BIM will be introduced into cells and treated with appropriate second-generation targeted therapy (e.g. Pan-HER inhibitors for EGFR mutant T790M cancers).

Summary of Results, Progress and Accomplishments: We have introduced pTREX BIM in low BIM cancers and have shown that this sensitizes cancers to TKIs. We are now in the process of performing similar studies in EGFR mutant lung cancers with T790M resistance mutations and impaired apoptosis to 3rd generation TKIs.

2I. Current objectives: Stain slides made from FFPE tissue derived from patients with EGFR mutant lung cancer specimens following radiographic evidence of resistance as identified by Dr. Sequist using methods previously described (Sequist et al, Science Translational Medicine, 2011). In conjunction, slides made from FFPE tissue derived from these patients prior to initial treatment will be stained.

Summary of Results, Progress and Accomplishments: As described above, 25 matched pre-treatment and post-resistant biopsies from patients with EGFR mutant NSCLC have been obtained and are currently being stained for BIM expression and we expected the results within 2-3 weeks.

2J. Current objectives: Stain slides made from FFPE tissue derived from patients with *ALK* translocated lung cancer specimens following radiographic evidence of resistance as identified by Dr. Shaw. In conjunction, slides made from FFPE tissue derived from these patients prior to initial treatment will be stained.

Summary of Results, Progress and Accomplishments: *ALK* positive cases have been collected. We are waiting for initial results with EGFR samples to make sure that results are high quality before committing the *ALK* samples for the same analysis.

2K. Current objectives: Analyze progression free survival (PFS) in relationship with BIM expression, as determined by IHC, comparing BIM levels of the treatment-naïve patient tissue with BIM levels of the patient following radiographic evidence of acquired resistance.

Summary of Results, Progress and Accomplishments: This subtask will be completed after the BIM expression analyses are complete and more mature clinical follow-up data is available.

Aim 3: Assess novel therapeutic strategies for cancers with low BIM expression that aim to increase BIM and thereby enhance response.

Task 3:

Current Objectives: Interrogate 200 female NU/NU mice for this task.

Summary of Results, Progress and Accomplishments: Please see below in section on Subtasks.

Subtasks.

3A. Current objectives: Determine if demethylase inhibitors increase free BIM and if co-treatment with demethylase inhibitors and TKIs re-sensitize low BIM expressing resistant cancers, identified previously and in Aim 2, to apoptosis in vitro.

Summary of Results, Progress and Accomplishments: These studies have not yet been initiated. We have focused significant efforts for objective 3D. Those results have been very impressive, leading to a clinical trial sponsored by CTEP and they have occupied more effort than we initially planned.

3B. Current objectives: Determine whether there are alterations at the BIM promoter causing epigenetic silencing at the BIM locus in resistant lung cancer models identified previously and in Aim 2.

Summary of Results, Progress and Accomplishments: We have performed epigenetic CHIP-seq studies on the BIM locus in cancers that lose BIM expression and are awaiting further results to inform how the BIM locus changes upon silencing. In one model, an EGFR mutant lung cancer resistant line made resistant to EGFR inhibitor in vitro, we have seen marked changes at the BIM promoter in the resistant line compared to the parental line. These changes have corresponded with a large increase in the

transcriptional repressor, ZEB1, in the resistant line. We have located ZEB1 binding sites in the BIM promoter, and short-hairpin knockdown of ZEB1 leads to de-repression of BIM in this cell line. We are awaiting results from further analyses at the BIM promoter in this cell line to determine if ZEB1 increase results in BIM promoter alterations causing silencing at the BIM locus in resistant lung cancer.

3C. Current objectives: Determine whether agents that work by reversal of epigenetic silencing (e.g. demethylase inhibitors) lead to de-repression of BIM in these cancers.

Summary of Results, Progress and Accomplishments: These studies have revealed that demethylase inhibitors de-repress BIM expression in a subset of cancers. We are currently aiming to understand the underlying mechanisms that will identify which cancers are regulated in this manner

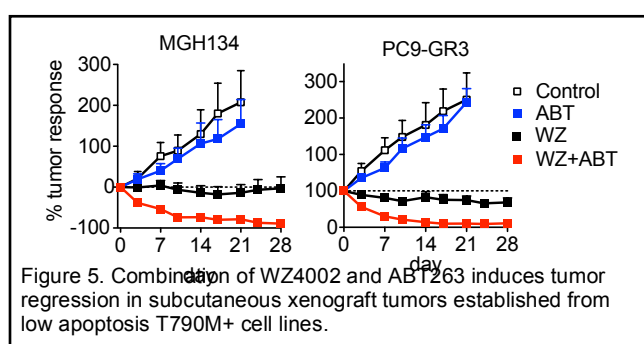
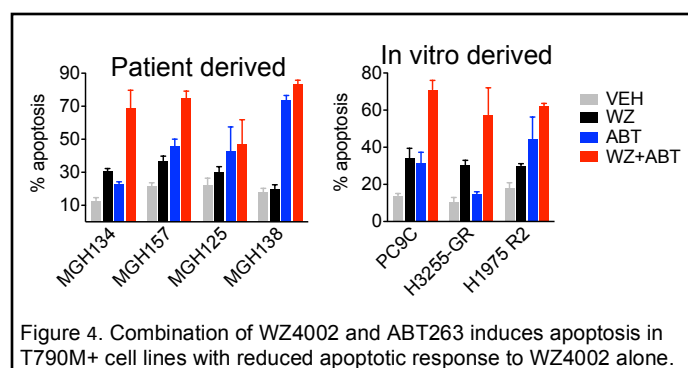
3D. Current objectives: Determine whether the amount of free BIM can be maximized in low BIM expressing resistant cancer models identified previously and Aim 2, by addition of ABT-263, through immunoprecipitation analysis of Bcl-2 family member proteins.

Summary of Results, Progress and Accomplishments: We have determined that free BIM can be enhanced by addition of ABT-263.

3E. Current objectives Determine whether BH3 mimetics sensitize low BIM expressing lung cancers identified previously and Aim 2 to TKIs to apoptosis.

Summary of Results, Progress and Accomplishments: In the majority of T790M resistant cell lines (both in vitro generated as well as patient-derived cell lines from resistant patients) with decreased apoptotic response to irreversible EGFR inhibitors, ABT263 (BH3 mimetic) sensitizes to treatment with WZ4002 (irreversible EGFR inhibitor with T790M inhibition) in vitro, leading to increased apoptotic response and decreased long term viability (Figures 4 and 5).

These findings are the basis for a new clinical trial combining ABT263 with AZD9291. Our data was presented to a national committee of lung cancer experts convened by NCI-CTEP and was chosen from several potential ideas as a promising concept. A clinical trial proposal was designed by these experts (including Dr. Engleman) and presented to the NCI Investigational Drug Steering Committee, who voted to proceed with the trial. Hence, a national CTEP-sponsored trial will be rolling out in 2015. This trial is the direct result of the studies supported by this DOD award.



3F. Current objectives: Use the pharmaceutical strategy that does in fact re-sensitize low BIM

expressing cancers to TKIs, and treat these cancers with this combination in vivo by xenografting female NU/NU mice.

Summary of Results, Progress and Accomplishments: We have tested the combination of ABT263 + WZ4002 in xenograft models of in vitro acquired resistance (PC9) as well as patient-derived cell line from resistant tumor (MGH134) and demonstrated that this combination induces dramatic tumor regressions (Figure 5).

4.KEY RESEARCH ACCOMPLISHMENTS:

- Intrinsic loss of apoptotic response appears to be a distinct mechanism contributing to acquired resistance and not mutually exclusive with commonly clinically observed genetic clinical mechanisms of resistance such as T790M
- Cancers with T790M and suppression of apoptotic response may be less sensitive to subsequent therapy with next generation irreversible EGFR inhibitors
- Cancers with loss of apoptotic response can be resensitized to TKI treatment by use of BH3 mimetics
- **New clinical trial combining ABT263 with AZD9291 is planned based on these results**

5.CONCLUSION:

We have established that a subset of resistant cancers that developed T790M have decreased apoptotic response to irreversible EGFR inhibitors in vitro and in vivo. The changes in these cells that underlies the loss of apoptotic response may involve BIM in some cases but overall appears to be more complex. We have determined that the combination of BH3 mimetics plus irreversible EGFR inhibitors is effective against many of these cancers in preclinical studies. These data have provided rationale for planned upcoming clinical trials investigating the combination of navitoclax (ABT263) with irreversible EGFR inhibitors.

6.PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

A manuscript describing the combination of ABT263 and irreversible EGFR inhibitors is currently being developed.

7.INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8.REPORTABLE OUTCOMES:

A manuscript describing the combination of ABT263 and irreversible EGFR inhibitors is currently being developed and will be the basis of an upcoming clinical trial

9.OTHER ACHIEVEMENTS:

We have developed 28 cell lines from patients with acquired resistance to EGFR inhibitors and 20 from patients with acquired resistance to ALK inhibitors. A subset of these cell lines will be published shortly.

10. REFERENCES: b/a

11. APPENDICES: n/a