Award Number: W81XWH-17-1-0136

TITLE: Thymine DNA Glycosylase as a Novel Target for Lung Cancer

PRINCIPAL INVESTIGATOR: Alfonso Bellacosa, M.D., Ph.D

CONTRACTING ORGANIZATION: The Research Institute of Fox Chase Cancer Center 333 Cottman Avenue, Philadelphia, PA 19111

REPORT DATE: July 2018

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 *(DD-MM-YYYY)*	2. REPORT TYPE	3 DATES COVERED
July 2018	Annual	1Jul2017 - 30Jun2018

4. TITLE AND SUBTITLE
Thymine DNA Glycosylase as a Novel Target for Lung Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0136

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alfonso Bellacosa, M.D., Ph.D./Timothy J. Yen, Ph.D.

E-Mail: Alfonso.Bellacosa@fccc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Research Institute of Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, Pennsylvania 19111
E-Mail: osr@fccc.edu

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 purpose of this research is to develop novel therapeutics for lung cancer (LC) based on targeting the dual DNA repair and epigenetic factor Thymine DNA Glycosylase (TDG). In principle, inhibiting TDG may both disrupt DNA repair and the cancer epigenetic state of LC cells, thus achieving a therapeutic effect that enhances the actions of existing chemotherapies and is complementary to current epigenetic therapy agents.

In the current reporting period, we showed that TDG knockdown in the non-small lung cancer (NSLC) cell lines A549 and NCI H1993 causes cell cycle alteration and reduction of clonogenic capacity. In order to pursue mechanistic studies of this anti-cancer effect, we have generated derivatives of these LC cell lines that express doxycycline-inducible shTDG constructs. In preparation of future experiments with anonymized patient-derived cell lines and mouse xenograft experiments, we have submitted the relevant HRPO and ACURO forms, respectively.

15. SUBJECT TERMS
DNA methylation, DNA demethylation, DNA repair, epigenetic therapy, Thymine DNA Glycosylase, cancer cell killing, single-agent therapy, combination therapy, combination index

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unlimited	Unclassified		12	19b. TELEPHONE NUMBER *Include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

DOD Annual Report

Principal Investigators: Alfonso Bellacosa & Timothy Yen
Institution: The Research Institute of Fox Chase Cancer Center
Grant Number: W81XWH-17-1-0136

INTRODUCTION: The purpose of this research is to develop novel therapeutics for lung cancer (LC) based on targeting the dual DNA repair and epigenetic factor Thymine DNA Glycosylase (TDG). In principle, inhibiting TDG may both disrupt DNA repair and the cancer epigenetic state of LC cells, thus achieving a therapeutic effect that enhances the actions of existing chemotherapies and is complementary to current epigenetic therapy agents.

KEYWORDS: DNA methylation, DNA demethylation, DNA repair, epigenetic therapy, Thymine DNA Glycosylase, cancer cell killing, single-agent therapy, combination therapy, combination index

ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of this project are: (Specific Aim 1) to characterize the mechanisms by which TDG inhibition facilitates growth arrest, senescence and cell death; and (Specific Aim 2) to explore candidate TDG inhibitors as single agents and in combination with existing chemotherapies and epigenetic therapies.

What was accomplished under these goals?

We have previously shown (ms. under review) that *TDG* knockdown in melanoma cell lines produces an anti-cancer effect characterized by reduced proliferation and decreased clonogenic capacity. In the current reporting period, we tried to determine whether this effect can be reproduced in lung cancer (LC) lines. We tested our hypothesis on A549 and H1299, two non-small cell lung cancer (NSCLC) adenocarcinoma cell lines. Two methods were used for RNAi-mediated genetic knockdown of *TDG*; (1) lentiviral infection with a plasmid (pLKO.1) encoding an shRNA targeting *TDG*, named C8 (Cortellino et al. Cell, 146: 67-79, 2011), (2) transfection with a combined pool of siRNAs (SMARTpool, Dharmacon) targeting multiple regions of *TDG* mRNA.

In the shRNA method, we generated recombinant lentivirus using standard protocols, tested viral titer by assaying for efficiency of puromycin resistant cells at different dilutions of viral supernatants. Once titer was determined, cells were infected with C8 lentivirus or with control pLKO.1 empty lentiviral vector. Two days after infection, cells were selected in medium containing puromycin (1.0 micrograms/ml). Cells were harvested on post-infection day 6 and lysates were prepared. Western blot analysis of the lysates revealed that the C8 lentivirus can knock down TDG expression in both A549 and H1299 cells (Fig. 1A). In the siRNA method, the two NSCLC cell lines, seeded on a 6-well plate, were transfected with 20nM of siRNA. Cells were harvested and lysates prepared at time-points ranging from 2 to 6 days. Western blot analysis of those lysates confirmed persistent reduction of TDG expression for up to 6 days (Fig. 1B). Thus, we have confirmed that both methods can independently induce TDG knockdown. This pertains to Aim 1 Subtask 1.

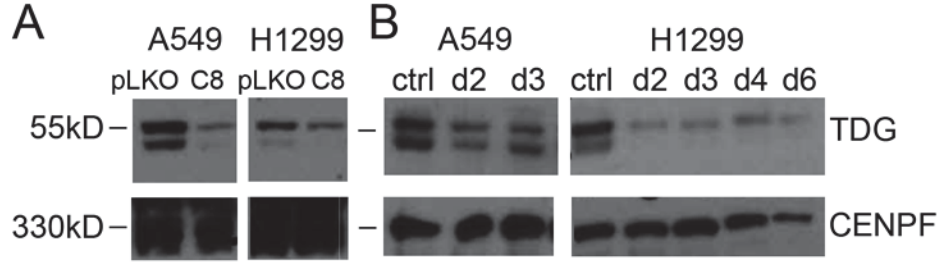

Figure 1. Western blot analysis showing effective TDG knockdown in A549 and H1299 cells by lentiviral shRNA infection (A) and siRNA transfection (B). CENPF is a loading control.

To assess the impact of TDG knockdown on colony-forming ability of NSCLC cell lines, A549 and H1299 cells infected with C8 lentivirus or with control pLKO.1 empty lentiviral vector were selected with puromycin on day 2, passaged for 7-8 days in puromycin, and seeded in triplicate on 12-well plates at 600 cells/well. After 10 days, the cells were fixed in 10% methanol/acetic acid for 1 minute, then stained with 4% Crystal Violet in 20% EtOH for 10 minutes. Wells were destained with 30% ethanol/10% acetic acid and individual colonies were counted. The results revealed that TDG knockdown leads to an approximately 4-fold reduction in colony-forming capacity of both cell lines, confirming the inhibitory effect of TDG knockdown on cell proliferation (Fig. 2). We independently verified the lentiviral knockdown data with siRNA transfections. A549 and H1299 cells were transfected with TDG SMARTpool siRNA for 3 days before seeding (triplicates) for colony formation. The normalized clonogenic data were as follows: A549 control = 1.0, A549 TDGsiRNA = 0.49; H1299 control = 1.0, H1299 TDGsiRNA = 0.62. The higher survival rate of the TDG siRNA-transfected cells could be due to the fact that the effects of the siRNA were not as long lasting as with lentivirus. The transfected siRNA gets diluted out over time and TDG expression becomes restored. With this caveat in mind, the trend we saw with the siRNA experiment is consistent with the lentiviral knockdown of TDG. This completes Aim 1 Subtask 2.

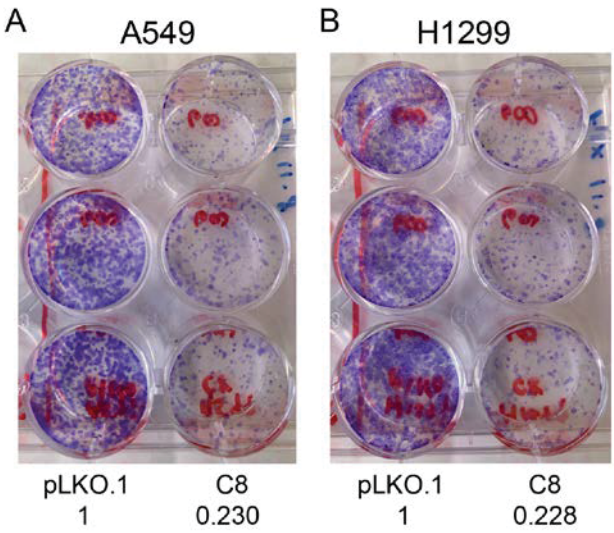

Figure 2. Clonogenic capacity of A549 (A) and H1299 (B) cells infected with TDG shRNA lentivirus (C8) or control pLKO.1 vector. All the experiments were performed in triplicate. Average number of colonies were normalized to 1.0 (pLKO.1 samples).

In order to pursue mechanistic studies of this anti-cancer effect, we have just initiated the cell cycle analysis of A549 and H1299 cells infected with control (pLKO) and TDG lentiviruses (C8), similar to conditions used for clonogenic data. Our preliminary experiment was to infect with the viruses, start puromycin selection, and then collect cells on days 3 and 4 post selection. The cell cycle profiles on day 4, as analyzed by flow cytometry (FACS), show a slight but noticeable decrease in the S phase population for both cell lines (Fig. 3). This is our first experiment, and we will continue along this line of analysis by taking longer time points (as we do not yet know how long it takes for the effects of TDG knockdown to be manifested). Separately, we are in the process of generating derivatives of these LC cell lines that express doxycycline-inducible shTDG constructs. In preparation of future experiments with anonymized patient-derived cell lines and mouse xenograft experiments, we have submitted the relevant HRPO and ACURO forms, respectively.

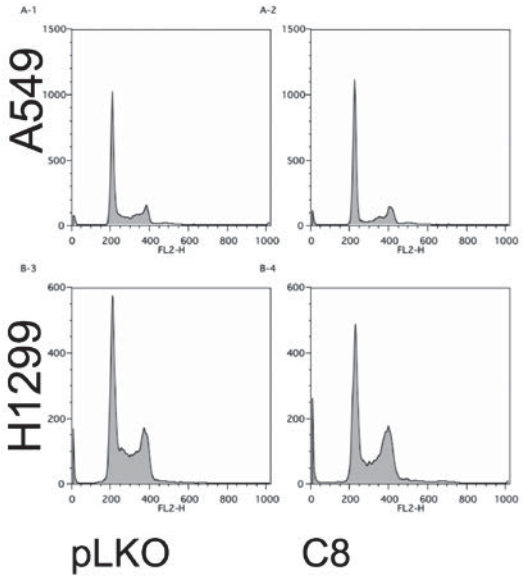

Figure 3. Fluorescence-activated cell sorting (FACS) of propidium iodide-stained A549 and H1299 cells, showing that TDG knockdown induces a slight decrease in S phase cells.

Because we had to identify commercial sources of siRNAs and optimize transfection conditions, it took us longer than expected to obtain siRNA data and prepare and resubmit HPRO and ACURO forms, causing an overall delay on the SOW. However, now that the siRNA procedure has been established, we are confident we can move quickly to complete all the Tasks of the SOW within the expected time frame.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we plan to publish the results showing that TDG knockdown causes cell cycle arrest and senescence of LC cell lines. We also plan to assess the mechanisms by which TDG knockdown has such anti-cancer effects on LC cell lines, to complete Aim 1. Finally, for the final report, we plan to investigate the role of TDG inhibitors as single agents or in combination with agents used in the clinic against LC, to complete Aim 2.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The impact of the finding that TDG knockdown causes cell cycle arrest and reduction of clonogenic capacity of LC cells is significant, because it indicates that a new paradigm of lung cancer therapy can be introduced, by which targeting TDG may have a dual detrimental effect on lung cancer by both reducing the DNA repair capacity and disrupting the epigenetic state of cancer cells. In particular, the presumed consequence of TDG knockdown (DNA hypermethylation) is diametrically opposed to that of demethylating agents, immediately raised the innovative possibility of synergy between TDG inhibitors and demethylating agents.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

PRODUCTS:

Publications, conference papers, and presentations

Journal publications. Nothing to Report.

Books or other non-periodical, one-time publications. Nothing to Report.

Other publications, conference papers, and presentations. Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	***A. Bellacosa, M.D, Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.00*
Contribution to Project:	Preparation and submission of internal IRB protocols and HRPO forms. Preparation and submission of internal IACUC protocols and ACURO forms. Design and implement TDG knockdown experiments and experiments with TDG inhibitors on lung cancer cell lines.
Funding Support:	N/A
Name:	***T. Yen, Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.00*
Contribution to Project:	Design and implement TDG knockdown experiments. Create inducible vector for shTDG. Assess viability of lung cancer cells after TDG knockdown by colony formation assay. Analyze candidate TDG inhibitors on lung cancer cell lines.
Funding Support:	N/A
Name:	***R. Tricarico, Ph.D.***
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	11.00
Contribution to Project:	Preparation of biological reagents for the project, including control and shRNA TDG lentiviruses. Tissue culture of lung cancer cell lines. Supply TDG inhibitors and test them on lung cancer cell lines.
Funding Support:	57.9% salary support from Avery Fellowship

Name:	***J. Hittle***
Project Role:	Technical Specialist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.00
Contribution to Project:	Technical support such as tissue culture, produce biological reagents for the project, conduct TDG knockdown experiments and analyze lysates for knockdown. Performing clonogenic assay.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Please see attached updated Other Support for key personnel. Changes are marked with a line in the right hand margin.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

APPENDICES: Not applicable.

Other Support

Bellacosa, Alfonso

Remaining salary support from institutional sources.

CURRENT

4100077072 (PI: Bellacosa) 1/1/2017 - 12/31/2018 15.0%
PA DOH CURE $120,337 1.80 calendar
Maintenance of Genomic and Epigenomic Stability at CpG Sites
The major goals of this project are: 1) To evaluate the role of TDG in imprinting; and 2) To evaluate the role of TDG in hematopoiesis and leukemogenesis.
Procuring Contracting/Grants Officer: Susan Guy, HRO, Rm. 833 Health & Welfare Bldg., 625 Forster St., Harrisburg PA 17120, 717-547-3711

W81XWH-17-1-0136 (PI: Bellacosa / Yen) 7/1/2017 - 6/30/2019 10.0%
DOD $163,717 1.20 calendar
Thymine DNA Glycosylase as a Novel Target for Lung Cancer (Multi-PI)
The major goals of this project are to: 1) Characterize the mechanisms by which TDG inhibition facilitates growth arrest, senescence, and cell death; and 2) Conduct translational studies of candidate TDG inhibitors as single agents, and in combination with existing chemotherapies and epigenetic therapies.
Procuring Contracting/Grants Officer: Amanda Carrera, 820 Chandler St., Fort Detrick, MD 21702, 301-619-2108

COMPLETED

R21 CA191956

OVERLAP

None

Other Support

Yen, Timothy J.

Remaining salary support from institutional sources.

CURRENT

W81XWH-17-1-0136 (PI: Bellacosa / Yen) 7/1/2017 - 6/30/2019 10.0%
DOD $163,717 1.20 calendar
Thymine DNA Glycosylase as a Novel Target for Lung Cancer (Multi-PI)
The major goals of this project are to: 1) Characterize the mechanisms by which TDG inhibition facilitates growth arrest, senescence, and cell death; and 2) Conduct translational studies of candidate TDG inhibitors as single agents, and in combination with existing chemotherapies and epigenetic therapies.
Procuring Contracting/Grants Officer: Amanda Carrera, 820 Chandler St., Fort Detrick, MD 21702, 301-619-2108

P30 CA006927 (PI: Fisher) 8/12/2016 - 7/31/2021 10.0%
NIH Salary only 1.20 calendar
Comprehensive Cancer Center Program at Fox Chase
The major goal of this Cancer Center Support Grant is to provide partial salary support for professional personnel, including senior and program leadership, administration, planning and evaluation, and developmental funds, as well as support for 5 established peer-reviewed Research Programs, 12 Shared Research Resources and 2 Support Elements.
Procuring Contracting/Grants Officer: Candace Cofie, 9609 Medical Center Dr., Bethesda, MD 20892, 240-276-6317

COMPLETED

R21 CA191956
R21 CA182651

OVERLAP

None

Other Support

Tricarico, Rossella

Dr. Tricarico is no longer at Fox Chase Cancer Center. We are actively recruiting a To Be Named Research Associate to replace Dr. Tricarico's efforts.