Award Number: W81XWH-20-1-0499

TITLE: Senescence Escape as a Mechanism of Tarceva Resistance in Retinoblastoma-Negative, EGFR-Positive NSCLC and Therapeutic Strategies to Circumvent

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REPORT DATE: OCTOBER 2021

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2021	Final	07/01/2020 - 06/30/2021

4. TITLE AND SUBTITLE

Senescence Escape as a Mechanism of Tarceva Resistance in Retinoblastoma-Negative, EGFR-Positive NSCLC and Therapeutic Strategies to Circumvent

5a. CONTRACT NUMBER
W81XWH-20-1-0499

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Albert Einstein College of Medicine, Bronx NY 10461

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development 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

Approximately one third of NSCLC patients have EGFR-mutant (+) disease, for which EGFR inhibitors (EGFRi: e.g. osimertinib, afatinib, erlotinib) are FDA-approved, first-line therapies. We demonstrated that clinically relevant concentrations of EGFRi induces senescence in EGFR+ lung cancer cell lines, and fibroblasts; the latter potentially leading to interstitial lung disease and pulmonary fibrosis. We established conditions for osimertinib-induced senescence and characterized the cell cycle regulatory pathways associated with this form of dormancy. Senescent cells were resistant to continued EGFRi therapy but sensitive to known senolytic therapeutics including navitoclax, cardiac glycosides and histone deacetylase inhibitors. Investigator-initiated studies revealed additional senolytic activities of receptor tyrosine kinase inhibitors and translation inhibitors. EGFRi-induced senescence cells were resistant to lentiviral mediated retinoblastoma (*RB*) ablation; therefore, the effect of *RB* loss on senescence and escape could not be determined. Senescent EGFR+ cells resume proliferation 2-3 weeks after osimartinib withdrawal. We posit that EGRFi-mediated senescence and subsequent escape induces genomic alterations associated with osimertinib resistance in EGFR+ cells. To investigate this, we are analyzing the genomic landscape of proliferating versus senescent EGFR+ cells. Furthermore, ongoing studies are assessing the effect of senolytic consolidation therapy in mice bearing EGFR+ tumors following daily osimertinib to induce minimal residual disease.

15. SUBJECT TERMS

EGFR+ NSCLC, Osimertinib, senescence, senoltyic, drug resistance, tumor dormancy, retinoblastoma

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
UU	UU	UU	UU	8	19b. TELEPHONE NUMBER *(include area code)*

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

TABLE OF CONTENTS **PAGE NUMBER**

1. Introduction

A mechanism of resistance to EGFR inhibitors (EGFRi) in EGFR-positive (+), non-small cell lung cancer (NSCLC) is histologic transformation whereby resistant tumors acquire molecular and phenotypic traits of Small Cell Lung Cancer (SCLC). Genetic alterations associated with this transformation include loss of TP53 and RB function, activating PIK3CA mutations, and EGFR and c-MYC amplification. It has been demonstrated that EGFRi can induce senescence in EFGR+ cancer cells. This form of tumor dormancy poses risk of recurrence since senescent tumor cells can persist, eventually resuming proliferation by escaping senescence. The senescence state is associated with significant genomic instability and aneuploidy; *therefore, we posit that genomic alterations that occur during senescence can be transmitted to daughter cells, contributing to the EGFRi-resistant phenotype*. Specifically, we hypothesize that loss of RB function associated with EGFRi resistance is caused by genomic alterations that occur during senescence. This project sought to confirm that EGFRi inhibitors induce a senescent state associated with genomic alterations that could contribute to resistance. Recognizing the threat posed by persistent senescent tumor cells and the reality that EGFR+ patients relapse on EGFRi monotherapy, we also tested and identified senolytic therapeutics that can kill EGFRi-induced senescent cancer cells. Ongoing studies are testing intermittent dosing of senolytics in combination with EGFRi in mouse models of EGFR+ lung cancer to improve progression-free survival by attenuating senescent cell accumulation. This proposal is highly translational and has the potential to positively impact the survival and quality of life of military members facing a lung cancer diagnosis.

2. Keywords

EGFR mutant NSCLC, Osimertinib, Senescence, Tumor dormancy, Drug resistance, Senolytics, Retinoblastoma, Histologic transformation.

3. Accomplishments

MAJOR GOALS

Task 1 - Prove that EGFR inhibition induces senescence in EGFR+ NSCLC cancer cell lines.

MILESTONES: Establish that (a) erlotinib induces stable senescence in vitro and in vitro, and (b) that genetic ablation of RB modulates the rate of senescence escape.

Milestone (a) has been **completed (100%)** for cell-based studies. *In vivo* studies in mouse models of EGFR+ cancer are ongoing (20% complete). Milestone (b) has not been achieved due to technical challenges caused by an inability to perform lentiviral infections in EGFRi-induced senescent cells, and / or loss of senescent cells following siRNA-RB genetic modulation.

Task 2 – Characterize genomic alterations that occur during senescence and senescence escape in EGFR+ NSCLC.

MILESTONES: (a) Contrast the genomic landscape of proliferating EGFR+ cells versus, EGFRi-mediated senescent cells, versus RB-deficient cells that escape from senescence. (b) Investigate whether senescence escape via RB silencing promotes a molecular genotype comparable to SCLC.

Milestone (a) **is 30% complete**. Genomic DNA has been extracted from EGFRi-induced senescent cancer cells, although the quality and quantity of the recovered material has not been suitable to generate a library for whole genome sequence, and RNA-seq analysis. Current effort is focused on increasing the number of senescent cells to improve yield and quality of recovered nucleic acids. Milestone (b) has not been achieved due to technical challenges caused by an inability to perform lentiviral infections in EGFRi-induced senescent cells, and / or loss of senescent cells following siRNA-RB genetic modulation.

Task 3 – Test the vulnerability of erlotinib-induced senescent cancer cells and cells that have escaped senescence to death-inducing therapies (SENOLYTIC molecules).

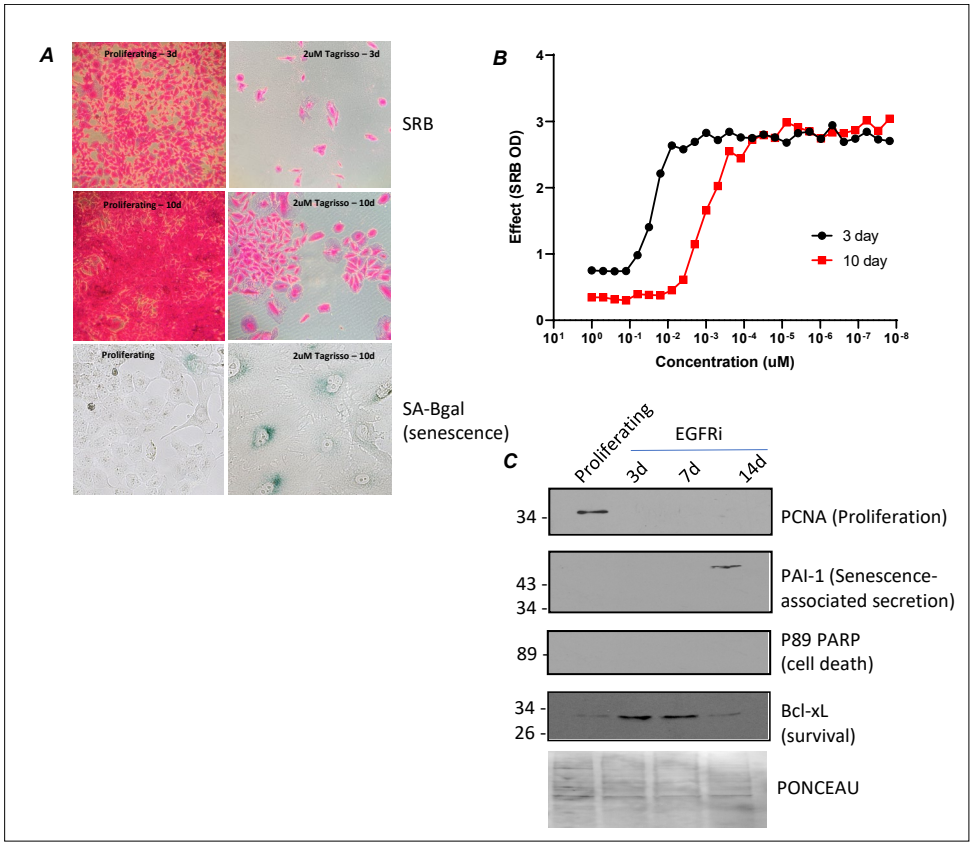
MILESTONES: Determine the vulnerability of EGFRi-induced senescent, and SCLC-transformed EGFR-mutant lung cancer cells to therapeutics that target the molecular vulnerabilities of senescent cancer cells and RB-deficient cancer cells.

Due to technical challenges caused by an inability to perform lentiviral infections in EGFRi-induced senescent cells, or perform post-infection analyses, this task was modified to determine therapeutic vulnerabilities of EGFRi-induced senescent cancer cells and **has been completed (100%)**. *In vivo* studies to evaluate the anti-tumor efficacy of senolytic consolidation therapy in combination with intermittent EGFRi therapy have not been initiated.

MAJOR ACTIVITES - during the funding period related to (a) verifying that EGFRi causes senescence in cancer cells following prolonged treatment, (b) characterizing the rate of spontaneous escape from EGFRi-induced senescence (resumption of proliferation), (c) testing the response of EGFRi on senescent cancer cells to determine efficacy, and (d) evaluating the anti-cancer efficacy of potential senolytics in EGFR+ senescent cancer cells. Other major activities that were unsuccessful, or are partially complete include, (e) infecting EGFRi-induced senescent cancer cells with human RB1 siRNA to test the hypothesis that loss of RB accelerates escape from senescence, and (f) extraction of genomic DNA and RNA from EGFRi-induced senescent cancer cells to evaluate genome-wide alterations that may occur upon senescence induction.

SPECIFIC OBJECTIVES - Goals for the funding period are to prove that EGFR inhibition induces senescence in EGFR+ NSCLC cancer cell lines: to determine if senolytic therapies cause cell death in EGFRi-induced senescent NSCLC , and to investigate if EGRFi-mediated senescence and senescence escape cause genetic alterations, particularly those involving the RB or TP53 locus.

SIGNIFICANT RESULTS / KEY OUTCOMES - We have established the conditions for osimertinib-induced senescence in EFGR+ NSCLC. Cells were exposed to EC80 concentrations of osimertinib q2 days x5 and senescence confirmed using standard methods (absence of cell death, prolonged cessation of proliferation and increased secretory phenotype – **FIG 1**). Thus, we have confirmed that senescence is an outcome of prolonged Osimertinib therapy in EFGR+ NSCLC. Data is shown for HCC827. Additional data has also been generated for an additional 2 EGFR+ cell lines, H1975 and H1650 (not shown).



CELL LINE	% Senolytic Effect (72h) *Osimertinib-Induced senescence*		
	Osimertinib	**Afatinib**	**Erlotinib**
H1650 (exon 19, p53 WT)	6	20	6
HCC827 (exon 19, p53 mut)	20	ND	ND
H1975 (L858R, T790M, p53 mut)	20	ND	ND

As shown in the **Table**, the senolytic activity of various EGFRi (EC80 concentrations) was determined (ND=not done). EGFRi-induced senescent cancer lines were largely refractory to EGFR inhibitor therapy, **calling into question the clinical utility of continuous EGFRi-therapy in patients**.

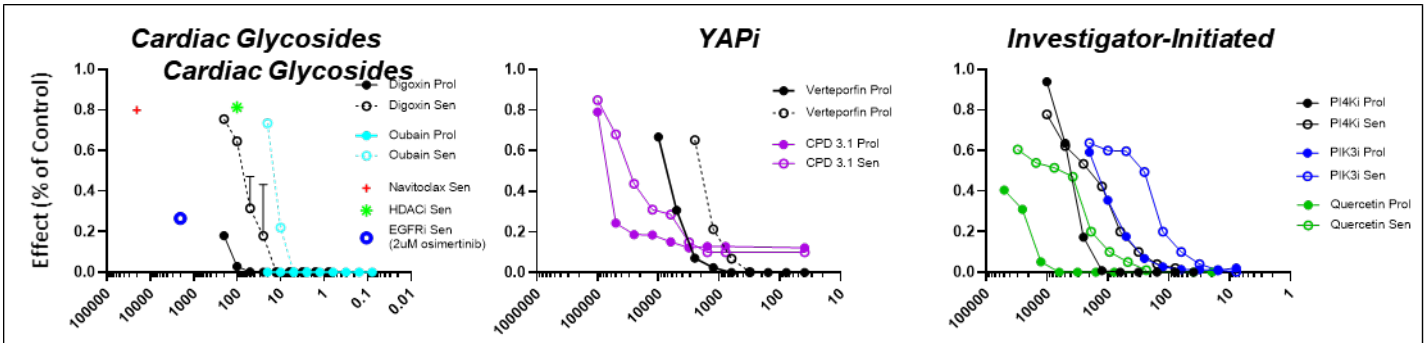
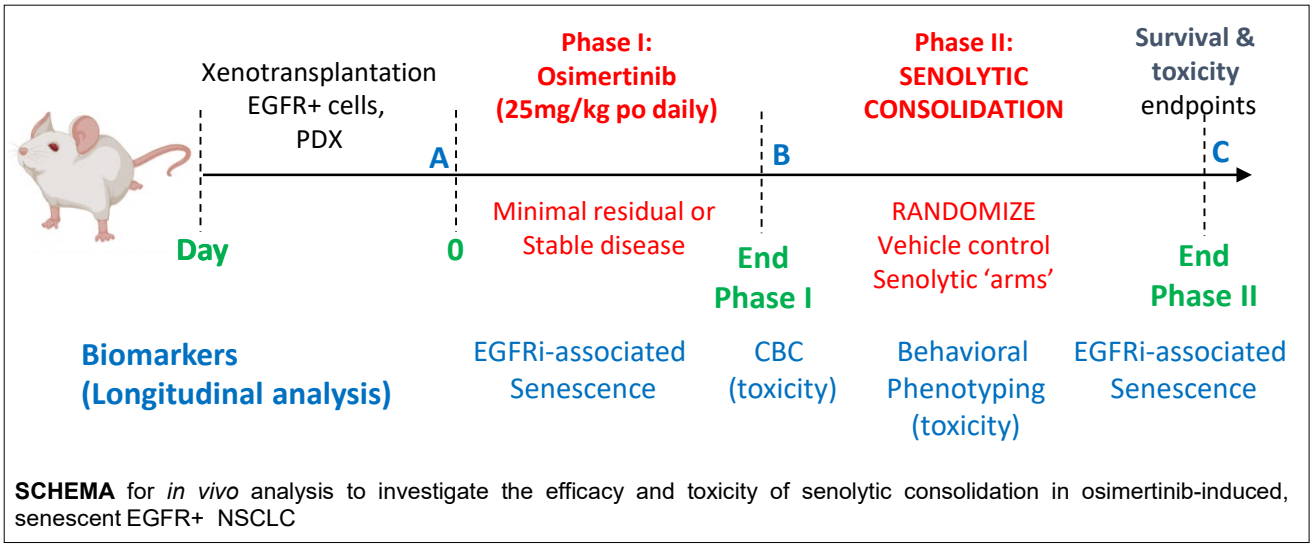


FIG 2: Senolytic Efficacy in Osimertinib-Induced Senescent EGFR+ Cells (HCC827). Cells were exposed to 2uM Osimertinib q2dx5 to induce senescence. 12 day-old cells were then seeded onto 96-well plates to test the efficacy of various molecules for senolytic activity over 72h (dashes lines = senescence). Efficacy was also evaluated in proliferating HCC827 cells (solid lines = proliferating). Effect was determined as % of DMSO controls and 1 = 100% cell kill (senolysis). Efficacy of known senolytics (navitoclax and HDACi) are shown.

We evaluated the senolytic efficacy of several classes of molecules in senescent EGFR+ cells (**FIG 2**). Consistent with other reports, CGs, YAP inhibitors and the flavonoid quercetin (currently in clinical evaluation with dasanatib) demonstrated senolytic activity in senescent EGFR+ cells. These data have also been generated for 2 additional EGFR+ cell lines, H1975 and H1650 (not shown).

GOALS NOT MET - We hypothesize that 2nd-line senolytic consolidation will improve survival and circumvent osimertinib resistance (via eradication of senescent cells). A xenograft model will be used to test several arms of senolytic candidates selected from our pilot studies in EGFR+ senescent cells (**FIG 2**). Daily osimertinib will be used to induce minimal residual disease (see **schema**). Mice will be randomized into cohorts for senolytic consolidation (1-week: recommended MTD). Depending on efficacy and toxicity, osimertinib dosing will resume for 1 additional week followed another week of senolytic therapy. Treatments will be discontinued and mice monitored for tumor re-growth and toxicity. *Data from this experiment will be curated to select the most efficacious senolytic(s) and schedule for additional evaluation in 2 EGFR+ PDX models, as well as monitoring cumulative toxicity from histology, CBC, behavioral phenotyping and body condition metrics*.



SCHEMA for *in vivo* analysis to investigate the efficacy and toxicity of senolytic consolidation in osimertinib-induced, senescent EGFR+ NSCLC

We have met with challenges in our efforts to characterize genomic alterations that occur during senescence and senescence escape in EGFR+ NSCLC, and genetic modulation of retinoblastoma in EGFR+ senescent cells. Genomic DNA has been extracted from EGFRi-induced senescent cancer cells, although the quality and quantity of the recovered material has not been suitable to generate a library for whole genome sequence, and RNA-seq analysis. Current effort is focused on increasing the number of senescent cells to improve yield and quality of recovered nucleic acids. We have also met with considerable technical challenges genetically manipulating

senescent cells, and or performing activating a CrispR-RB transgene once cells become stably senescent. Efforts are ongoing to circumvent these challenges.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT – Nothing to report

RESULTS DISSEMINATED TO COMMUNITIES OF INTEREST - Nothing to report

PLAN DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS – This is the final report. Experiments described in the "goals not met" section will be performed. Alternate funding sources will be sought to continue these important studies.

4. Impact

We seek to identify a specific phase of lung cancer treatment enriched for dormant (senescent) tumor cells . This phase could be vulnerable to cellular and / or genomic programming: thereby permitting these tumor cells to persist and resume proliferation could propel clinically recalcitrant disease. The study seeks to confirm that this senescence dormancy phase exists and will test the efficacy of senolytic therapeutics that can eradicate these cancer cells during treatment. We posit that intermittent dosing of senolytics could be beneficial in EGFR+ lung cancer and improve progression-free survival **by limiting senescent cell accumulation**. Based on the clinical reality that EGFR+ patients relapse on EGFRi monotherapy, we will extrapolate these studies to test in vivo, with the long-term goal of translating to clinical evaluation in our Bronx population, and in particular individuals diagnosed with EGFR+ lung cancer following active military service and potential exposure to carcinogens (e.g. fire pit exposure). Overall, the research outlined here is highly translational and has the potential to positively impact the survival and quality of life of military members facing a lung cancer diagnosis.

IMPACT ON OTHER DISCIPLINES - Nothing to report

IMPACT ON OTHER DISCIPLINES - Nothing to report

IMPACT ON TECHNOLOGY TRANSFER - Nothing to report

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY - Nothing to report

5. Changes/Problems **-** Nothing to report

Changes in approach, actual or anticipated problems or delays, Changes that had a significant impact on expenditure, significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents; Significant changes in use or care of human subjects; Significant changes in use or care of vertebrate animals and Significant changes in use of biohazards and/or select agents

6. Products

Publications, conference papers, and presentations **-** 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

7. Participants & Other Collaborating Organizations

Individuals who have worked on the project

Name:	*Hayley McDaid, Ph.D.*
Project Role:	*PI*
Nearest person month worked:	*1.2 calendar months*
Contribution to Project:	*Project leader: supervised other personnel: analyzed data: compiled reports.*
Funding Support:	*NCI, BCRF (other)*

Name:	*Francisco Marques, Ph.D.*
Project Role:	*Post-doctoral research associate*
Nearest person month worked:	*9 calendar months*
Contribution to Project:	*Performed experiments: Analyzed data: Compiled Figures.*
Funding Support:	*NCI (other)*

Change in the active other support of the PD/PI(s) or senior/key personnel - Nothing to report

Other organizations involved as partners - Nothing to report

8. Special Reporting Requirements - Nothing to report

9. Appendices - Nothing to report