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Development of Antidepressants as Novel Agents to Treat Small Cell Lung Cancer

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### ABSTRACT

Small cell lung cancer (SCLC) is an aggressive neuroendocrine subtype of lung cancer with high mortality. We used a systematic drug repositioning bioinformatics approach querying a large compendium of gene expression profiles to identify candidate U.S. Food and Drug Administration (FDA)-approved drugs to treat SCLC. We found that tricyclic antidepressants and related molecules potently induce apoptosis in both chemonaïve and chemoresistant SCLC cells. The candidate drugs activate stress pathways and induce cell death in SCLC cells, at least in part by disrupting autocrine survival signals involving neurotransmitters and their G protein-coupled receptors. These experiments identify novel targeted strategies that can be rapidly evaluated in patients with neuroendocrine tumors through the repurposing of approved drugs.

### SUBJECT TERMS

SCLC, small cell lung cancer, tri-cyclic anti-depressants
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INTRODUCTION
The identification of therapeutic approaches for the treatment of cancer is an arduous, costly, and often inefficient process. Drug repositioning, which is the discovery of new indications for existing drugs that are outside their original indications, is an increasingly attractive mode of therapeutic discovery. In addition to saving time and money, an advantageous aspect of drug repositioning is that existing drugs have already been vetted in terms of safety, dosage, and toxicity. Therefore, repurposed candidate drugs can often enter clinical trials much more rapidly than usual. The primary goal of the research funded by this award was to validate candidate drugs identified through a computational repurposing approach against small cell lung cancer and to uncover the mechanisms of action of these drugs.

KEYWORDS
SCLC, small cell lung cancer, drug repositioning, tri-cyclic antidepressants, GPCR signaling

OVERALL PROJECT SUMMARY
Small cell lung carcinoma (SCLC) is a neuroendocrine form of lung cancer. SCLC patients have a 5-year survival of less than 5%. This dismal survival rate has remained the same for the last 30 years and ~200,000 patients die every year worldwide from SCLC, emphasizing the need for the development of novel therapeutic approaches against this aggressive cancer subtype. The idea behind this proposal was to use a drug repositioning approach to identify and validate FDA-approved drugs that can be repurposed to treat SCLC. A unique aspect of our strategy was that we used a novel bioinformatics-based drug-repositioning pipeline based on the analysis of thousands of gene expression profiles experiments.

We had already conducted a preliminary bioinformatics analysis and identified candidate drugs that are predicted to inhibit the growth of SCLC. Among these candidate drugs are inhibitors of G-protein coupled receptors (GPCRs) including anti-depressant molecules; those are novel candidate inhibitors of SCLC. Our objective was to test the effects of these drug candidates on mouse and human SCLC cells in culture and in vivo and to identify one or two top candidates, as well as to uncover the mechanisms of action of these candidates. Before the beginning of the proposal, we found that tricyclic antidepressants (TCAs) and related molecules potently induce apoptosis in both chemonaïve and chemoresistant SCLC cells. The candidate drugs activate stress pathways and induce cell death in SCLC cells, at least in part by disrupting autocrine survival signals involving neurotransmitters and their G protein-coupled receptors.

Specific tasks and timeline (defined in the initial Statement of Work):
- Task 1 (from now to the beginning of the funding period, including the time required for review and approval processes for mouse studies): expansion and maintenance of the mouse colony to continually generate mice with the appropriate genotypes, including NSG immunocompromised mice. This task was achieved towards the end of year 1, but we kept the mouse colony active for new experiments in year 2.
- Task 2 (first few months): experiments in mouse and human SCLC and control cell lines to test the effects of the four top candidate drugs. This task was achieved by the end of year 1.
- Task 3 (months 1-9): while the first cohorts of mice developing are aging, we will perform short-term experiments in allografts and xenografts transplanted under the skin of NSG mice. The effects of the drugs will be quantified using markers of proliferation, apoptosis, and differentiation. This task was achieved by the end of year 1.
- Task 4 (months 6-18): 4-6 months after Ad-Cre infection, tumor development will be measured in Rb/p53/p130 mutant mice using the luciferase reporter and mice will be treated with the drugs. Histopathological analysis will be performed, using markers of proliferation and differentiation. This task was achieved by the end of year 1.
- Task 5 (months 18-24): we will complete the experiments in Aim 1 in cell lines and mice. This task was largely achieved by the end of year 1 and completed in year 2.

- Task 6 (months 6-12): once we have identified the best cell lines and the top candidate drugs, we will perform the initial experiments to investigate the mechanisms of action of these drugs. In particular, we will measure cell death, cell proliferation, ROS production. This task was achieved by the end of year 1.

- Task 7 (months 6-18): we will perform all the experiments for the second part of Aim 2, investigating the role of various GPCRs in promoting survival in SCLC cells. This task has been largely achieved.

- Task 8 (months 18-24): we will use that time to apply for additional funding and to initiate a novel series of experiments (including gene expression profiles, and the analysis of signaling pathways). This task has been largely achieved by the end of year 1.

As noted above, we had made significant progress within a year regarding most, if not all the tasks originally listed in the SOW. Thus, in year 2, we expanded that scope.

KEY RESEARCH ACCOMPLISHMENTS

- We validated tricyclic antidepressants such as Imipramine as inducers of cell death in SCLC (Aim 1 of the research proposal)

- We determined that TCA treatment inhibit PKA signaling in SCLC cells and leads to increased stress signals eventually resulting in cell death (Aim 2 of the research proposal)

Summary of the first year of funding (data corresponding to these findings can be found in the progress report for the first year)

To briefly summarize what we did in year 1:

1) We performed a second drug repositioning analysis in silico, using novel gene expression datasets for human SCLC in the literature and improved computational methods. This analysis generated a second list of candidate drugs to test (these candidates are now coined “perturbagens”). Interestingly, we found drugs interacting with signaling pathways that we previously identified as critical in SCLC cells in our Cancer Discovery study \(^1\) (e.g. JNK). It is also well known that PI3K \(^2\) and Bcl-2 \(^3\) are playing a role in SCLC cells (Table 1) (See Figure 2 in Progress Report Year 1 for the initial validation for preliminary data on Selamectin).

2) We performed a whole-genome screen \(^4\) to identify genes whose loss-of-function would prevent the induction of cell death by Imipramine; in this screen, every gene in the genome has been disrupted by several retroviral integrations and top candidates are genes for which many independent integrations are shown (See Figure 3 in Progress Report Year 1). Notably, among the top candidates, a number of regulator of cell death have been identified (e.g. BCL2L1, also known as BCLXL, or BAX, or PMAIP1 also known as NOXA). Very interestingly, PMAIP1 was initially identified as “Phorbol-12-Myristate-13-Acetate-Induced-Protein-1”, which means it is induced by the phorbol ester PMA, itself identified in the new drug repositioning experiment (top hits in Figure 1). Another interesting candidate

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Table 1: Computational drug repositioning – top candidate drugs from the second iteration.
from this experiment is SLC3A2, which is also known as CD98; it is a solute carrier that can transport amino acids in cells, such as phenylalanine, tyrosine, leucine, arginine and tryptophan. It has been found in a gene fusion event in lung cancer and could play a role in SCLC progression; it may also play a role in metastasis. However, its role in the survival of SCLC cells is completely unknown and we have begun to generate reagents to study its function in SCLC; one interesting observation is that SLC3A2 interacts with the glucose transporter GLUT1, which raises the possibility that its loss-of-function may affect tumor metabolism.

3) In parallel to these new mechanistic efforts, we explored the possibility that an alternative mechanism for how TCAs kill neuroendocrine cancer cells: a possible lysosomotropic mechanism in which the drugs may accumulate in lysosomes, blocking their function, thereby leading to cell death. The best known lysosomotropic agent is chloroquine (because its deprotonated form is more membrane-permeable than its protonated form, it is trapped in lysosomes). Our experiments to test this possibility indicate that there may be indeed some induction of cell death in culture in SCLC cells through this mechanism, but this is only seen at high doses of the drug (around 20 hours after treatment) and we do not know yet if similar mechanisms may be at play in tumors in vivo (See Figure 4 in Progress Report Year 1).

4) Finally, we pursued our analysis of the possible role of PKA downstream of TCAs in SCLC cells: PKA was identified as a key enzyme downstream of TCAs in our previous analysis since PKA activators completely blocked the inhibitory effects of TCAs. This aspect was further developed in the second year (See Figure 6 in Progress Report Year 1).

Summary of the second year of funding (related to the initial Statement of Work):

Following up on our results and a clinical trial at Stanford University: Desipramine and Imipramine

We have worked with Dr. Joel Neal at Stanford to implement and analyze a phase IIa prospective clinical trial that was designed to identify efficacy with a primary endpoint of observing at least one partial response among ten previously treated patients (pts) with small cell lung cancer (SCLC) or high grade neuroendocrine tumors (HGNET). To be eligible, pts were required to have failed at least one prior chemotherapy for metastatic small cell lung cancer or HGNET (Ki-67 >= 20% or >= 20 mitoses/10 HPF). Previously treated brain metastases were allowed. Treatment with desipramine began at 75 mg nightly with weekly visits for dose escalation as tolerated to a ceiling of 450 mg daily (max FDA-approved dose is 300 mg). Tumor measurements were done at baseline and every 8 weeks with responses determined using RECIST 1.1 criteria. A total of six pts were enrolled, 3 with SCLC, and 3 with HGNET (lung, rectal, and pancreatic). All but one had advanced stage at the time of diagnosis, and 3 had CNS metastases. Prior to entry, pts received a median of 3.5 prior systemic treatments (range 1-9). Max stable doses were 300 mg (1 pt), 150 mg (2 pts), 75 mg (1), and two pts were unable to tolerate any stable dose. Reasons for discontinuation included drug-related grade 3 colon pseudo-obstruction, unrelated GI bleed, and grade 1-2 drug related dizziness, confusion, and somnolence. Of the 6 pts, none received continuous therapy for a full 2 months, but 4 had evaluable scans subsequent to treatment discontinuation, all of which demonstrated progressive disease. The other two pts entered hospice after functional decline. Though numbers are small in this analysis, median clinical and radiographic progression free survival was 1.2 months (range 0.2-3.3) and median overall survival from study entry was 2.7 months (range 1.3-5.6). Although preclinical evidence was promising, no clinical or radiographic benefit was observed by using desipramine to treat SCLC and HGNET, so this trial was closed early. The tolerable doses achieved may have been inadequate for antitumor efficacy. Drug repositioning has the potential to accelerate oncology drug development, but physicians should be cautious when translating preclinical results into practice. Please note that these experiments with patients were completely funded by an internal grant from the Stanford Cancer Institute to Dr. Joel Neal. No funds from the DoD grant was used for these studies, we just mention this work here because it is directly related to the proposed work (a natural extension).
The one interesting fact from this trial is that we had switched to Desipramine in patients based on the recommendation of a psychiatrist that Desipramine would have fewer side-effects than Imipramine and on very early results we had obtained in cell lines that Desipramine killed SCLC cells as much as Imipramine. However, based on the results in patients (lots of side effects and no obvious efficacy, even though very few patients were treated), we re-examined the effects of Desipramine in vivo. This took a lot of effort and we found that, in stark contrast to the Imipramine data, Desipramine had very little effect (and could even promote cancer, not significantly).

We also performed a survival curve on a mouse model of another neuroendocrine cancer (pancreatic neuroendocrine tumor model, which was also described in our Cancer Discovery paper and found to be very responsive to Imipramine). Here again, Desipramine had no inhibitory effect (Figure 2).

These results were very interesting because Imipramine and Desipramine are very similar in structure but have radically different biological activity in SCLC models. We are currently exploring the possibility that Imipramine and Desipramine may have very different metabolites in mice.

We went from bench to bedside to bench again and performed more MTT viability assays on multiple SCLC and NE cell lines to test low doses of imipramine (10mg/kg). Treatment with daily IP injections for 30 days at 10mg/kg did not lead to any obvious side effects in mice but did not have a significant inhibiton of tumor growth or a decrease in tumor burden (not shown). So reducing the dose may alleviate side effects but may also not have any anti-cancer effects – it’s probably not the right strategy.

**Mechanisms of resistance to TCAs.**
A second aspect that we have been working on is the possibility that SCLC cells may eventually become resistant to Imipramine. We used one human PDX model (H29 cells) and treated them ~50 days with Imipramine. We observed that the tumors began to be less sensitive to the drug after 4-5 weeks. We took these treated tumors and re-implanted them into new recipient mice and observed clear signs of resistance to treatment. We thought we could use these cells to better understand the mechanisms of action of the drug but when we placed these tumors in culture, they were not resistant any more to Imipramine (data not shown). Thus, resistance can occur but we do not understand how, even though our data suggest that it may arise from a non-cell autonomous mechanism in which cells in the microenvironment contribute to resistance. We are currently repeating these experiments with additional human tumors. It will be important in the future to understand the mechanism of resistance if Imipramine or similar drugs are used in SCLC patients.

Search for new drugs
We screened more drugs from the repositioning list, including verapamil, GW8510, Tyrphostin, Apigenin, Trazodone, and ifenprodil, and none of these drugs were as good as Imipramine in vitro. We decided not to test these drugs in vivo (Figure 4 and data not shown).

Mechanisms of action of TCAs
As discussed last year, in collaboration with the laboratory of Jan Carette at Stanford, we have performed a whole-genome screen to identify genes whose loss-of-function would prevent the induction of cell death by Imipramine; in this screen, every gene in the genome has been disrupted by several retroviral integrations and top candidates are genes for which many independent integrations are shown. We started knockdown some of these genes and performing MTT assays to look for rescue of the imipramine-induced cell death. Our results suggest that knocking-down Bax and PMAIP1 can partly rescue the effects of Imipramine – this is interesting but mostly confirmatory since these molecules are well known mediators of apoptotic cell death (Figure 5 and data not shown).

Another interesting candidate from this experiment is SLC3A2, which is also known as CD98; it is a solute carrier that can transport amino acids in cells, such as phenylalanine, tyrosine, leucine, arginine.
and tryptophan. It has been found in a gene fusion event in lung cancer⁶ and could play a role in SCLC progression; it may also play a role in metastasis⁷. However, its role in the survival of SCLC cells is completely unknown and we have begun to generate reagents to study its function in SCLC; one interesting observation is that SLC3A2 interacts with the glucose transporter GLUT1⁸, which raises the possibility that its loss-of-function may affect tumor metabolism. Our initial effort in knocking down slc3a2 in Kp1 and Kp3 cells did not yield to efficient knockdown (not shown). We are currently switching to more efficient knockout strategies such as CRISPR. We have generated successful CRISPR plasmids with gRNA against slc3a2 and transfected both mouse SCLC cells and sorted the GFP+ cells into single well sorts to expand individual CRISPR clones. We are still analyzing these data.

**PKA signaling in SCLC**

We have been pursuing our analysis of the possible role of PKA downstream of TCAs in SCLC cells: PKA was identified as a key enzyme downstream of TCAs in our previous analysis since PKA activators completely blocked the inhibitory effects of TCAs. This part of the project has been slowed by the long time it has taken to obtain mice in which a point mutation in PKA allows for its specific inactivation using a small molecule inhibitor and at the same time may allow to identify its targets (technology developed by the Shokat lab,⁹). However, to first confirm that PKA is indeed active in SCLC cells, we have used methods developed in the Shokat lab¹⁰-¹² to identify active kinases in cells (“inhibitor bead” approach¹³). These data were presented last year. We now have the mice ready for analysis. It is noteworthy that we obtained new funding from the DoD to study PKA in SCLC cells (Proposal Number LC140030, Award Number W81XWH-15-1-0250).

**Cancer stem cells: the ideal target population**

The cancer stem cell model has been proposed as a cellular mechanism that contributes to phenotypic and functional heterogeneity in tumors. This model assumes a hierarchical organization in which a subset of tumor cells, the cancer stem cell population, is responsible for sustaining tumorigenesis and establishing the cellular heterogeneity observed in a primary tumor. Cancer stem cells contribute to long-term tumor growth and have been proposed to be responsible for the maintenance and survival of tumors. We reasoned that it was important to identify cancer stem cells in SCLC because these would be the cells that we should target with Imipramine and other drugs. Although this was not part of the initial goal of this project, we felt that this was a key endeavor and we spent some of our effort towards this goal.

We identified a population of long-term, tumor-propagating cells (TPCs) in our genetically engineered mouse model of SCLC. This population, marked by high levels of the EpCAM and CD24 cell surface proteins, is also prevalent in human primary SCLC tumors derived from circulating tumor cells (CTCs). SCLC TPCs are numerous and highly proliferative but not intrinsically chemoresistant.

**Figure 6**: Mouse SCLC tumors contain a high fraction of cells capable of tumor-propagating cells in transplantation assays. A. Schematic representation of the workflow used to identify tumor-propagating cells (TPCs) in a pre-clinical mouse model of SCLC (TKO, Rb/p53/p130 mutant). B. Representative flow cytometry gating scheme of tumor cells isolated from SCLC tumors in Rb/p53/p130 mutant mice and stained with markers of cell death (7AAD), Lineage (CD45, CD31, and Ter119), CD24, CD44, and EpCAM (n>20). C. Extreme limiting dilution analysis (ELDA) of Lineage-negative (Lin−, bulk tumor cells), CD24High CD44Low EpCAMHigh and CD24High CD44Low EpCAMLow cells sorted from SCLC tumors in Rb/p53/p130 mutant mice and injected subcutaneously in NSG mice.
indicating that not all the clinical features of SCLC tumors can be linked to TPCs. Some of these data are summarized in Figure 6 (and data not shown). We have not yet been able to test the effects of Imipramine on these cells (we need to age more mice) but this will be an important goal in the future.

**PUBLICATIONS, ABSTRACTS, AND PRESENTATION**

**a) Publications:**


The grant was awarded August 1st, 2013, and the manuscript was resubmitted 2 weeks later and accepted another week after that, so there is some overlap between the funding time and the final acceptance of the paper, and we have used some of the funds from this award to complete the experiments.

Note that the study on cancer stem cells is under revision at Cell Reports. Note also that the clinical trial is being written up – we hope that two more papers will be published this year.

**b) Presentations (specifically on this project):**


Oct. 2014: invited speaker at the “Journées de Recherche Respiratoire (J2R)”, a symposium organized by the French Pneumology Society, Bordeaux, France.

April 2015: chairperson and organizer of a Recent Advances in Organ Site Research session on “Progress in Understanding Small Cell Lung Cancer” at the Annual Meeting of the AACR, Philadelphia.

April 2015: invited speaker at the IASLC Small cell lung cancer workshop, Memorial Sloan Kettering Cancer Center, NY.

August 2015: invited speaker and session chair, Models and Mechanisms of Cancer, Salk Institute, CA.

Sept. 2015: invited speaker at the 16th IASLC World Conference on Lung Cancer, Denver, CO.

**INVENTIONS, PATENTS, AND LICENSES**

Nothing to report

**REPORTABLE OUTCOMES**

As mentioned above, a clinical trial was implemented based on our results, led by Joel Neal: [https://clinicaltrials.gov/ct2/show/NCT01719861?term=joel+neal&rank=1](https://clinicaltrials.gov/ct2/show/NCT01719861?term=joel+neal&rank=1)

The results of the trial are still being written up.

**OTHER ACHIEVEMENTS**

Nothing to report
PUBLISHED STUDY

Nothing to report

APPENDICES

Abstract for last two oral presentations, as requested by the program officer Sheila Rowe (the NY meeting was very informal in the way we submitted the abstract).

OVERALL CONCLUSIONS

From these two years of funding, we have:

- identified new FDA-approved drugs that might be repositioned against SCLC. Our goal is to find funding to further test these new candidates.

- pursued the mechanisms of action of TCAs in SCLC and shown mostly that they work by inhibiting cell survival, which could suggest future combination therapies (maybe combination with drugs that affect the cell cycle more or could enhance the cell death). We have established a collaborative effort with Michael Ohlmeyer at the Mount Sinai School of Medicine for some medicinal chemistry approach to modify these compounds and hopefully find more active ones and elucidate their mechanisms.

- begun a new project on the role of PKA signaling in SCLC. This project is now funded by a new grant from the DoD.

- started studies on the most tumorigenic SCLC “cancer stem cells”, which may provide a better system to determine the effects of drugs in SCLC.
REFERENCES


Intra-tumor Heterogeneity in SCLC

Jing S. Lim¹, Nadine S. Jahchan¹, Julie George², Becky Bola³, Christopher Morrow³, Francesca Trapani³, Dian Yang¹, Pawel Mazur¹, Sandra Cristea¹, Caroline Dive³, Martin Peifer², Roman Thomas², and Julien Sage¹

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Small cell lung cancer (SCLC) is a neuroendocrine subtype of lung cancer characterized by a fast growth rate, extensive dissemination, and rapid resistance to chemotherapy. Survival rates are dismal and have not significantly improved in the past few decades. Sequencing the genomes of over 100 human SCLC demonstrates universal inactivation of p53 and RB and identified inactivating mutations in NOTCH family genes in 25% of tumors. Accordingly, we found that activation of Notch signaling in a pre-clinical SCLC mouse model dramatically reduces the number of tumors and extends the survival of the mutant mice. In addition to suppressing proliferation, active Notch inhibits neuroendocrine gene expression in SCLC cells. Thus, Notch plays a key tumor suppressive role in SCLC and strategies to re-activate Notch in SCLC tumors may be beneficial to patients. The possible role of Notch- and Notch+ cells in SCLC tumors during cancer progression and in response to chemotherapy will be discussed.

In parallel, we also identified a population of long-term tumor-propagating cells (TPCs) in SCLC. In contrast to other tumor types, SCLC TPCs are abundant, highly proliferative, and not intrinsically chemoresistant. SCLC TPCs are also characterized by elevated Myc activity compared to non-TPCs. Importantly, decreasing Myc activity in tumors back to levels similar to those found in non-TPCs using genetic and chemical means inhibits the long-term growth of SCLC tumors. These experiments provide a rationale for therapeutic strategies aiming at reducing Myc activity to specifically eradicate TPCs and prevent the maintenance and spread of this aggressive lung cancer type.

At the histological level, SCLC tumor cells are often viewed as homogeneous. These studies and previous studies (e.g. Calbo et al., Cancer Cell, 2011 – Berns lab) identify several levels of intra-tumor heterogeneity in SCLC, which may contribute significantly to SCLC aggressive nature and resistance to therapy.
Intra-tumor heterogeneity in SCLC

Julien Sage

Small cell lung cancer (SCLC) is a neuroendocrine subtype of lung cancer characterized by a fast growth rate, extensive dissemination, and rapid resistance to chemotherapy. Survival rates are dismal and have not significantly improved in the past few decades.

The group of Roman Thomas and Martin Peifer sequenced the genomes of over 100 human SCLC, which demonstrates universal inactivation of p53 and RB and identified inactivating mutations in NOTCH family genes in ~25% of tumors. Accordingly, we found that activation of Notch signaling in a pre-clinical SCLC mouse model dramatically reduces the number of tumors and extends the survival of the mutant mice. In addition to suppressing proliferation, active Notch inhibits neuroendocrine gene expression in SCLC cells. Thus, Notch plays a key tumor suppressive role in SCLC and strategies to re-activate Notch in SCLC tumors may be beneficial to patients (George, Lim, et al., in press).

At the histological level, SCLC tumor cells are often viewed as homogeneous. These studies and previous studies (e.g. Calbo et al., Cancer Cell, 2011 – Berns lab) have identified several levels of intra-tumor heterogeneity in SCLC, which may contribute significantly to SCLC aggressive nature and resistance to therapy. We will also discuss the existence and the role of several subpopulations of SCLC tumor cells involved in the long-term propagation of this cancer type, the rapid acquisition of chemoresistance, and metastasis, as well as kinase signaling networks, including PKA, involved in SCLC maintenance. A better understanding of the molecular underpinnings of these cellular heterogeneity may help identify novel therapeutic targets in SCLC.