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TITLE: Dual Targeting of Tumor-Initiating Cells in Small Cell Lung Cancer

PRINCIPAL INVESTIGATOR: Sarmishtha De, PhD

CONTRACTING ORGANIZATION: Cleveland Clinic,
Cleveland, OH 44195

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14. ABSTRACT

Small cell lung cancer (SCLC) has a dismal prognosis despite aggressive therapeutic approaches, and there is a clear need to develop more effective interventions. The role of tumor-initiating cells (TICs) in SCLC is largely unknown, although it is widely believed to be an important mechanism driving chemo-resistance in other cancers. Among cancers, SCLC is recognized for its rapid response to chemotherapy and equally rapid relapse. Thus, it is an ideal cancer in which to study TIC targeting, and drugs that selectively eradicate TICs offer great promise for treatment in this disease. Moreover, combinations of drugs will have more beneficial effects than a single agent.

Here we seek to develop an innovative and novel therapeutic regimen for SCLC by identifying synergistic combination therapies using two drugs, Rovalpituzumab tesirine (ROVA-T) and CBL0137 (CBL), both of which target SCLC TICs. ROVA-T (RT), a potent anti-cancer humanized antibody-drug conjugate, selectively targets delta-like protein 3 (DLL3), which is highly expressed in SCLC TICs. The experimental drug CBL has potent anticancer activity. CBL inhibits the histone chaperone Facilitates Chromatin Transcription (FACT), which is required for the expression of transcription factors that are essential for TIC maintenance. Thus, the TIC-targeting mechanisms of CBL and RT are entirely different, targeting two different proteins, FACT and DLL3 that are highly expressed in SCLC TICs and each is thought to control the tumor-initiating properties through different pathways. Furthermore, combination of TIC-targeting drugs with traditional chemotherapy may be especially effective in overcoming resistance. Chemotherapy preferentially targets non-TICs, which comprise the bulk of tumors, but spares self-renewing TICs, providing the rationale for our second approach; using CBL and RT together to synergize with the standard-of-care chemotherapeutic agent cisplatin, as a novel overall treatment strategy for SCLC. Our hope is that targeting both non-TICs and TICs simultaneously will eradicate the cancer more completely, reducing tumor burden and delaying or even preventing tumor recurrence.

15. SUBJECT TERMS

Small cell lung cancer, tumor-initiating cells, CBL0137, Rova-T, cisplatin

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
Not classified	Not classified	Not classified		2	19b. TELEPHONE NUMBER *(include area code)* Sarmishtha De, PhD

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INTRODUCTION:

Genomic profiling of SCLC is in its infancy, delaying the development of molecularly targeted therapies; so the most immediate therapeutic improvement against this cancer may depend on our ability to prevent or delay the emergence of chemo-resistance that accompanies traditional chemotherapy. We believe that targeting TICs will accomplish this goal. Therefore, our objective is to develop novel therapeutic approaches that counteract relapse by eradicating TICs. However, no drug is likely to be curative as a single agent and, therefore, we propose an innovative and novel therapeutic regimen for SCLC, by testing the potential synergistic combination of two TIC-targeting drugs, Rovalpituzumab tesirine (Rova-T) and CBL0137 (CBL). CBL and Rova-T target entirely different proteins, FACT and DLL3, both of which are highly expressed in SCLC TICs, predicting increased efficacy of the combination of these drugs towards TICs. Furthermore, combination of TIC-targeting drugs with traditional chemotherapy may be especially effective in overcoming resistance. Chemotherapy preferentially targets non-TICs, which comprise the bulk of tumors, but spares self-renewing TICs, providing the rationale for our second approach; using CBL and Rova-T together to synergize with the standard-of-care chemotherapeutic agent cisplatin, as a novel overall treatment strategy for SCLC. Our hope is that targeting both non-TICs and TICs simultaneously will eradicate the cancer more completely, reducing tumor burden and delaying or even preventing tumor recurrence. These studies will lead to novel therapeutic approaches in SCLC, and will provide key supporting data for rapid translation into the clinic. This grant has received a no-cost extension for a year to allow us to continue pursuing the aims, thus this represents only an annual report. Due to delay in the approval of material transfer agreement we received the drug Rova-T from the company in February, 2019, and therefore, we describe below our progress for last 6 months.

1. KEYWORDS:

Small cell lung cancer, SCLC, tumor-initiating cells, TICs, CBL0137, Rova-T, FACT, DLL3, cisplatin, combination therapy, patient-derived xenografts, PDXs, cell survival, tumor growth, stem cell transcription factors.

2. ACCOMPLISHMENTS:

What were the major goals of the project? What was accomplished under these goals? The goals are listed as the grant Specific Aims as stated in the approved SOW and are followed by relevant accomplishments.

Specific Aim 1: To test the hypothesis that combining Rova-T with CBL0137 is synergistic in killing SCLC TICS.

Major Task 1:

Subtask 1: Determine the synergistic effects of combining Rova-T (RT) with CBL in the TICs and non-TICs derived from SCLC cell lines.

To determine the synergistic effects of combining RT with CBL, we performed cell survival assays in the TICs and non-TICs previously isolated from SCLC cell lines, H82 and H526. The well-characterized TICs or non-TICs were treated with CBL or RT or control IgG as single agents, or in combination with RT and CBL for 72 h. Cell survival was determined using the CyQUANT Direct assay, which measures proliferation as well as cytotoxicity. We show that the combination of RT and CBL significantly decreased the cell survival in H82 and H526 TICs than to treatment with either drug alone (Fig.1A, B). However, the drug combination has no additive effect on the sensitivity to non-TICs compared to the single drugs alone (Fig. 1C, D), emphasizing the preferential targeting of TICs by these drugs.

Fig. 1

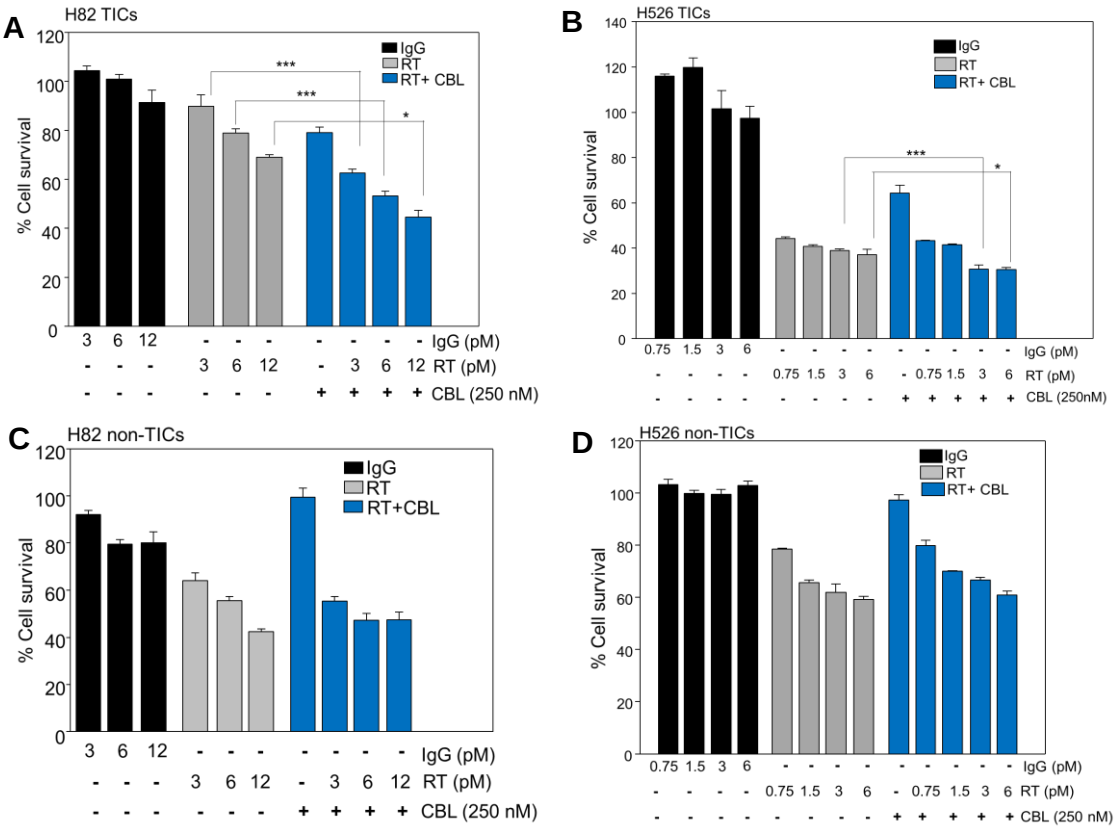

Fig. 1 SCLC TICs are more sensitive to combination of Rova T (RT) and CBL0137 (CBL) than single drugs alone. The cells were seeded at 3000 cells/well in black walled 96-well plates. Next day the cells were treated with IgG control or RT at different concentrations as shown in the figure or CBL or RT + CBL. The cell viability after 72 h of treatment was determined and normalized to untreated controls. The experiments were repeated thrice and each measurement was performed in triplicate. Results are represented by means ± SD. Data were analyzed using Student's *t* test. *P* values of <0.05 are considered statistically significant. *, $P< 0.05$, **, $P< 0.01$, ***, $P< 0.001$.

Subtask 2: Submit documents for ACURO approvals

We proposed to do experiments using mice. Therefore, we prepared and submitted the necessary documents to USAMRMC Animal Care and Use Review Office (ACURO) to get approval to perform experiments with animals. Our IACUC protocol number 2017-1863 was approved by ACURO on 12/07/2018 for the use of mice, and then we started the experiments we proposed using PDX models.

Subtask 3: Assess the synergy between RT and CBL using PDX model of SCLC

We determined the antitumor efficacy of RT combined with CBL, compared to single agents in a SCLC patient-derived xenograft (PDX) model. NOD/SCID mice were implanted with 50,000 PDX tumor cells and randomized into groups of 4 animals with average tumor volumes of 100 mm^3 per cohort. Mice were treated with vehicle control for CBL + IgG control, RT alone (1.8 mg/kg, i.p.,) or CBL alone (60 mg/kg, i.v., once/ week), or with combinations of RT with CBL. Tumor volumes were measured until they reach 1200 -1500 mm^3 in the vehicle-treated mice, at which time all groups were euthanized, and the tumors were removed. Tumor volumes (v) were calculated using the volume for a prolate spheroid: $v = 4/3 * \pi * a^2 * b$, where a = minor radius, b = major radius. Differences between groups were analyzed by Student's t-test. There was no significant reduction in the tumor growth in the mice treated with RT+CBL until day 50, compared to the single drug treated groups. However, tumor size started decreasing significantly ($p< 0.05$) after day 55 in the combo group (**Fig. 2A**). Since the vehicle treated group already reached the maximum size by that day, we sacrificed mice in all the groups. We excised the tumors from each group to determine the levels of SOX2, NANOG, OCT4 by qPCR, Western blotting and IHC, which we are planning to do soon.

In another experiment tumor-bearing mice (N=8) were treated with the vehicle controls and the drugs at the same dose as above, but in this experiment, the mice in each group were treated until it reached the maximum size of ~1200 mm^3. As shown in **Fig. 2B,** so far mice have been monitored for 102 days. RT in combination with CBL substantially inhibited tumor growth, compared to RT alone ($P< 0.05$) or CBL alone ($P< 0.05$), or vehicle control ($P< 0.05$). Mice treated with vehicle or single agents survived for 49 – 74 days, (**Fig.3**). The drug treatment was started on day 31. Importantly, one mouse is still alive in the combo group even after 102 days, bearing a very small tumor.

These results together indicate that RT in combination with CBL decreased SCLC tumor growth in SCLC PDX tumors, revealing a novel potent combination therapy for this cancer.

Fig.2A

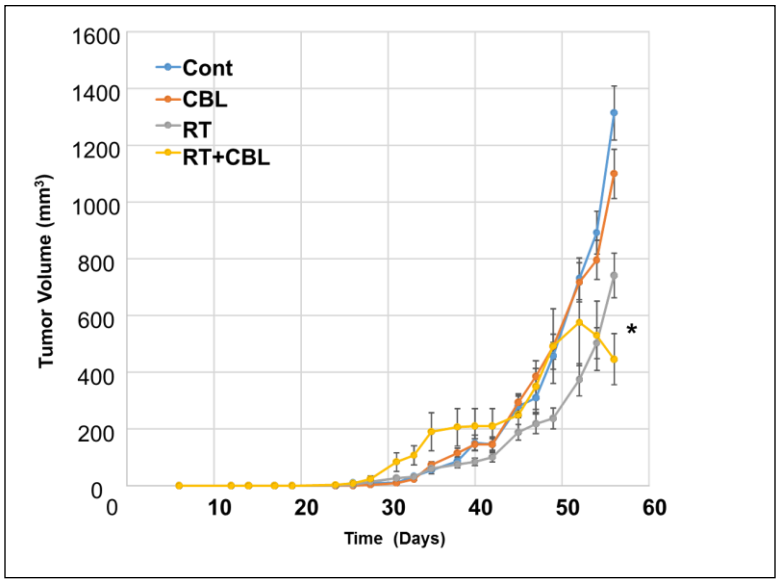

Fig. 2B

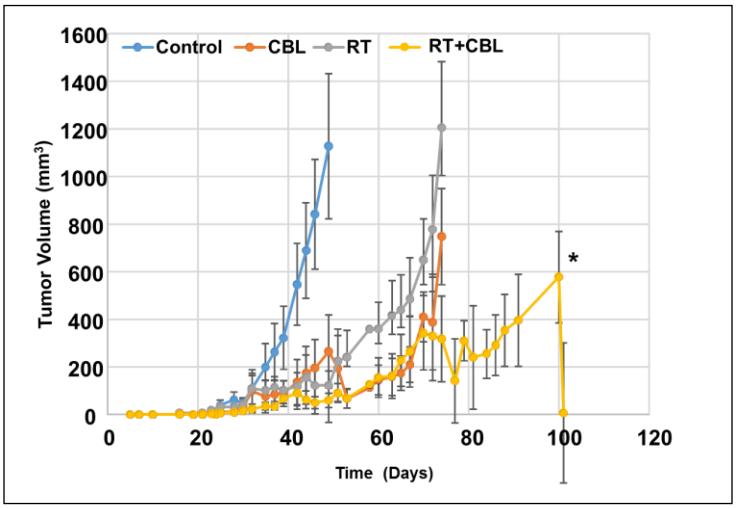

Fig.2. Tumor volume in mice after treatment with combination of RT and CBL. SCLC PDX tumor fragments (2 mm) were inoculated s.c. into the flanks of NSG mice. Once the tumors reached ~20 mm^3, the mice were randomized to treatment with vehicle + IgG control, CBL0137 (CBL), Rova-T (RT) , or RT + CBL. Tumors measured 3 times a week (**A**). The treatment was continued until the tumor volume reached ~1200 mm^3 in each group **(B)**. The results are represented as mean ± SE. * indicates $p<0.05$ versus the single drug treatment groups.

Fig. 3

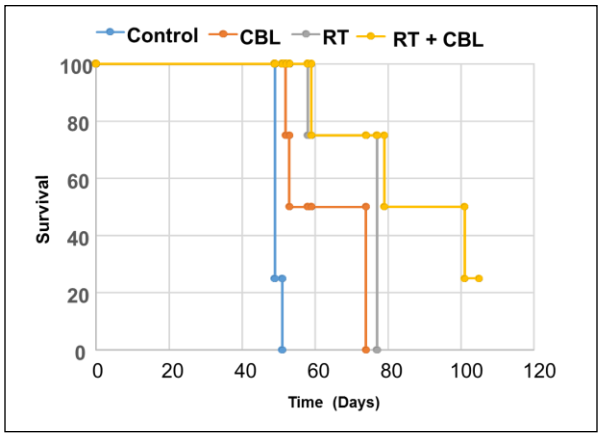

Fig.3. Survival of mice shown in Fig. 2B (*P<0.05* for combo vs single drugs alone)

Major Task 2:
Perform the in vivo limiting dilution assays

For in vivo limiting dilution study tumors were harvested from the above study (Fig.2A) and cell suspensions were prepared and inoculated subcutaneously in limiting dilutions (10^2 , 10^3 , 10^4 , 10^5) into naïve NSG mice, (4 mice/group x 2 inoculations/mouse, n=8 inoculations for each dilution). This experiment is still ongoing. The tumor size has been measured twice a week.

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?

Specific Aim 1:

i) We will determine the expression levels of TIC transcription factors SOX2, NANOG, and OCT4 in the cells by qPCR and Western analysis

ii) We will determine the expression levels of SOX2, NANOG and OCT4 by qPCR, Western blotting and immunohistochemistry in the tumor samples.

iii) To complete the on-going *in vivo* dilution assay.

iv) To determine the synergistic effects of combining RT with CBL in the TICs and non-TICs derived from additional SCLC cell lines

Specific Aim 2:

i) To determine the therapeutic effect of cisplatin in combination with Rova T and CBL in the SCLC cell lines and ii) in SCLC PDXs.

4. IMPACT:

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

Our findings together suggest that Rova T in combination with CBL0137 exerts a potent inhibitory effect on TICs derived from SCLC cell lines, significantly decreased tumor growth in PDXs, and increases the days of survival of mice.

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: Nothing to report

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

The only delay has been in our ability to obtain the drug Rova T from the company. It look a long time to get the approval of the material transfer agreement. However, we have been able to resolve that issue and are currently working on the project using the drug.

Changes that had a significant impact on expenditures
Nothing to report

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Sarmishtha De, PhD
Project role:	PI
Researcher identifier:	N/A
Nearest person months worked:	12
Contribution to project:	PI- Dr. De has worked oversight and direction.
Funding support:	This grant
Name:	Claire Coleman
Project role:	research technologist
Researcher identifier:	N/A
Nearest person months worked:	4
Contribution to project:	Ms. Coleman performed the tissue culture experiments
Funding support:	This grant
Name:	Daniel Lindner, MD, PhD
Project role:	Collaborator
Researcher identifier:	N/A
Nearest person months worked:	6
Contribution to project:	All mouse experiments were performed in collaboration with Dr. Lindner.
Funding support:	This grant
Name:	Afshin Dowlati, MD
Project role:	Collaborator
Researcher identifier:	N/A
Nearest person months worked:	12
Contribution to project:	Dr. Dowlati has provided research advice, and reagents.
Funding support:	N/A
Name:	George Stark, PhD
Project role:	Collaborator
Researcher identifier:	N/A
Nearest person months worked:	12
Contribution to project:	Dr. stark has provided advice on all aspects of this research.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS
Nothing to report

9. APPENDIX:

Revised SOW for the extended time of one year.

STATEMENT OF WORK – 09/30/2019
PROPOSED START DATE Aug 15, 2018

Site 1: Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland, OH 44195
PI: Sarmishtha De, PhD

Specific aim 1: To test the hypothesis that combining ROVA-T (RT) with CBL0137 (CBL) is synergistic in SCLC TICs	**Timeline (Months)**	
Major Task 1: To determine the expression levels of TIC transcription factors SOX2, NANOG, and OCT4 in the cells by qPCR and Western analysis.		
Subtask 1: Isolate RNA and perform qPCR	1-2	Sarmishtha De
Subtask 2: Make protein lysates for Western blot analysis	2-3	Sarmishtha De
Major Task 2: To determine the expression levels of SOX2, NANOG and OCT 4 by qPCR, Western blotting and immunohistochemistry in the tumor samples.		
Subtask 1: Isolate RNA from tumors and perform qPCR	2-4	Sarmishtha De
Subtask 2: Make protein lysates from tumors for Western blot analysis	3-5	Sarmishtha De
Subtask 3: Process the tumor samples and perform immunohistochemistry	2-5	Sarmishtha De Imaging core
Major task 3: To complete the on-going *in vivo* dilution assay:	1-5	Tumor core Sarmishtha De
Major task 4: To determine the synergistic effects of combining RT with CBL in the TICs and non-TICs derived from additional SCLC cell lines	6-10	Sarmishtha De

Milestone(s) Achieved: Obtain ACURO approval		
Milestone(s) Achieved: Milestone(s) Achieved: Determination of the synergistic effects of combining Rova T and CBL0137 in SCLC tumor-initiating cells in vitro and in vivo		
Specific Aim 2: To test the hypothesis that combining RT and CBL increases the therapeutic efficacy of Cisplatin (CP)		
Major Task 1 Subtask 1: Determine the therapeutic effect of cisplatin in combination with RT and CBL in the SCLC cell lines. Subtask 2: Determine the therapeutic efficacy of cisplatin in combination with RT and CBL in SCLC PDXs	1-5 2-12	Sarmishtha De Tumor core