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TITLE: Endoplasmin: A Novel Therapeutic Target and Potential Marker of Chemoresistance

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CONTRACTING ORGANIZATION: The Ohio State University, Columbus, OH

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our proposed study is to identify endoplasmin protein expression as a predictive marker associated with OC chemoresistance and identification of these pathways as potential therapeutic targets. Utilizing an endoplasmin small molecule inhibitor we plan to assess chemotherapy treatment using in vitro and in vivo studies with mice models. Our proposed research has the potential to identify novel the mechanisms of endoplasmin-mediated chemoresistance and targeting ENPL might serve to revert tumors to a chemosensitive state on a cellular level, therefore, improving treatment effect and prognosis/overall survival.

15. SUBJECT TERMS

Cancer Biology, Target Therapy and Biomarkers

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1. INTRODUCTION

Mortality rates from ovarian cancer (OC) are the highest among all gynecologic cancers and current therapies to treat the disease have failed to improve survival rates. Thus, there is a *critical need* to identify novel targets for alternative therapies for OC to improve outcomes. To effectively treat OC, it is important to: (i) Identify predictive markers associated with chemoresistance and (ii) elucidate the pathways involved in intrinsic chemoresistance to identify novel therapeutic targets. With these targets objectives in mind, we propose to identify the association of endoplasmin (ENPL) with platinum-resistant OC. ENPL is a member of a family of adenosine triphosphate (ATP)-metabolizing molecular chaperones that have roles in stabilizing and folding other proteins localized to melanosomes and the endoplasmic reticulum (ER). While ENPL expression has been demonstrated to play a critical role in immune modulation, cancer, and chemoresistance, the exact molecular pathways by which it acts remain unknown. Our current preliminary study demonstrated that ENPL expression in the membrane fraction is highly elevated in platinum resistant OC cell lines and patient samples. The hypothesize of our proposed study is that overexpression of endoplasmin contributes to chemo-resistance and that targeting endoplasmin using novel small molecule compounds will facilitate more successful treatment of the disease. To test this hypotheses, the following specific aims are proposed:

Specific Aim 1: To identify the role of ENPL expression in chemosresistance and progression OC. Our *working hypothesis is that* OC cells expressing ENPL expel carboplatin through exosome secretion and that OC cells with high levels of ENPL will be resistant to platinum treatment.

Specific Aim 2: To evaluate the effects of inhibiting ENPL on the efficacy of carboplatin treatment using *in vitro and in vivo* mouse models. Our *working hypothesis* is that inhibiting ENPL by small molecule inhibitors (DAP-1 or DAP-2) will significantly increase the efficacy of carboplatin (CP).

2. KEY WORDS

Ovarian Cancer

Platinum resistance

Endoplasmin

Exosomes

Biomarker

Small molecule inhibitor (DAP-1)

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this study is to evaluate a novel protein, Endoplasmin, as a potential therapeutic target in chemoresistant ovarian cancer

What was accomplished under these goals?

We have identified the significance of key findings in SA1

(i) ENPL plays a key role in chemo-resistant;
(ii) Developed a MFD chip for exosome isolation in cancer cells;
(iii) Exosomes are highly elevated in platinum resistant OC cells;
(iv) ENPL expression as a marker of platinum resistance.
(v) ENPL inhibitor suppress tumor growth.

Specific Aim 1: To identify the role of endoplasmin (ENPL) expression in chemosresistance and progression OC.
1a) Prepare forms for approval of Animals use and protocols involved.
Milestone # 1 Animal use approval (Year 1: month1-2): **Completed 100%.**

1b) Prepare IRB forms for approval of human sample use and protocols involved.

Milestone # 1 human sample use approval (Year 1: month1-3): **Completed 100%**

Approach 1. To determine the role of ENPL in OC cell survival, proliferation, and drug resistance.

Milestone # 1. Confirm and correlate the ENPL expression to Platinum resistance by ENPL knockdown (KD) or overexpression (OE) in chemo resistant cells and sensitive ovarian cancer cell lines (Year 2: 1 to 3 months). **Completed 100%**

Approach 2. To determine the mechanism of OC chemoresistance by ENPL. **Approach 1**: Assessment of cells' sensitivity to carboplatin expressing wild type, knock down and over expression ENPL or MRP2 in OC sensitive or resistant cell lines.

Milestone # 2. Depletion of ENPL in WT chemoresistant cells impacts the MRP2 expression, analyze the carboplatin sensitivity in either ENPL or MRP KD cells (Year 2: 3 to 4 months). **Completed 100%**

Approach 3 & **4.** To determine if ENPL regulates exosome secretion through MRP2 in OC cells.

Milestone # 3 Confirm the role of ENPL-mediated exosome secretion through MRP2 in OC and further identify ENPL, carried in exosomes, can confer cisplatin resistance to a cisplatin sensitive background (Year 2: 4 to 5 months). **Completed 100%**

Approach 4. To evaluate the clinical significance of ENPL expression as a marker of platinum resistance and survival in OC patient samples.

Milestone # 4. This study will confirm the prognostic value of ENPL as a biomarker for chemoresistance, disease progression, and prediction of potential treatment response (Year 1: months 7 to 12). **Completed 100%.**

Milestone # 5. Evaluate the efficacy of ENPL inhibitor in combination with carboplatin in three different mice modes including immunocompetent and PDX mice model; the synergistic effects of ENPL inhibitor and CP

evaluate vivo mice studies based on the ENPL inhibition, suppression of OC tumor growth and apoptotic proteins expression in mice tissues (Year 2: months 8 to 12). **Completed 100%.**

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

We have training for Gynecology Oncology Clinical fellows and Undergraduate pre-medical students in this proposed project in vitro study.

Publication: **Draft 1 is preparing based on SA1 and SA2 proposed study and being currently finalizing the results.**

RESULTS

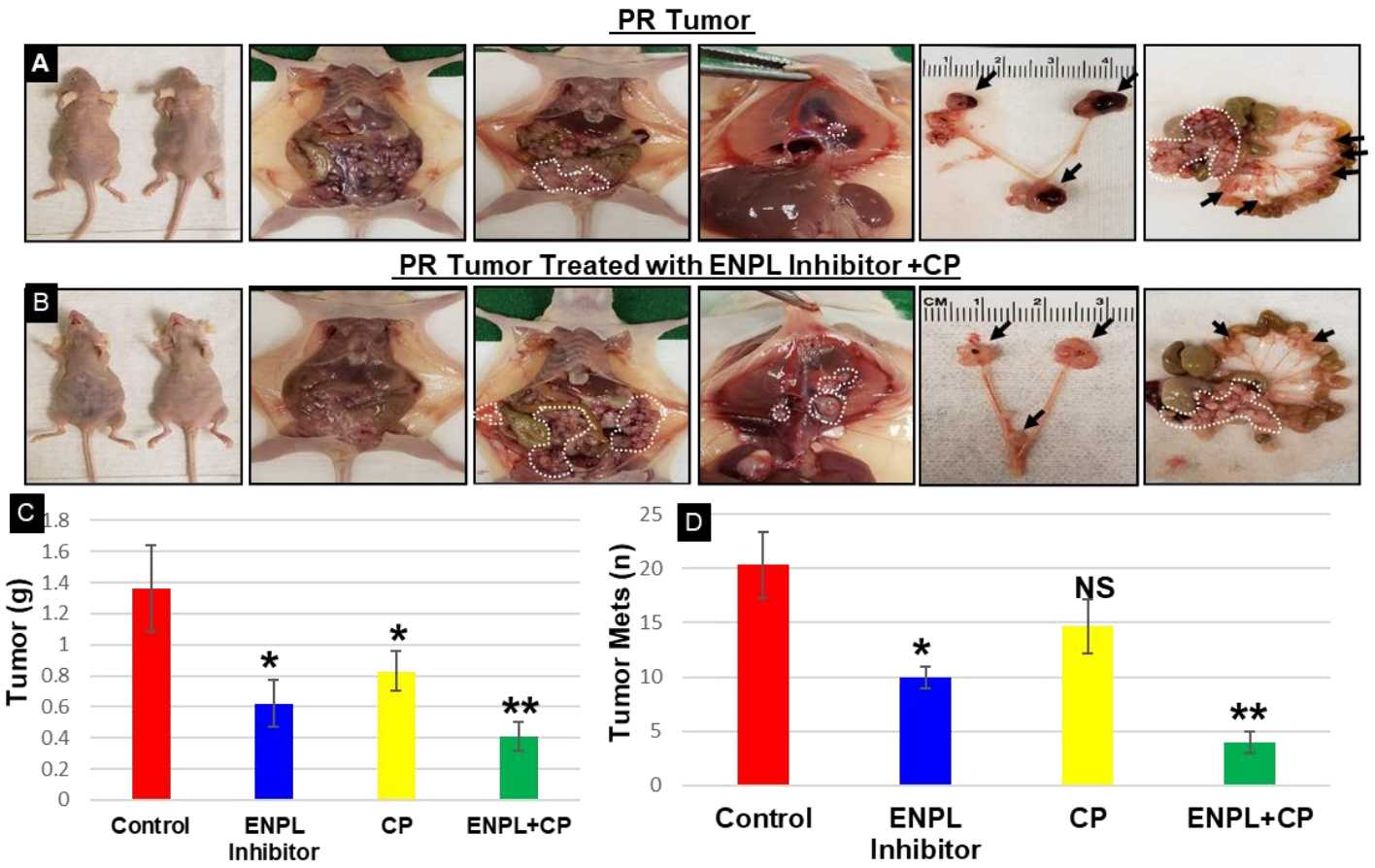
1. ENPL knockdown or inhibition by ENPL inhibitor, restores the CP sensitivity in OC *in vivo* mice model. TR-127 ENPL KD cells ($1x10^6$) were injected into the ovarian bursa (orthotopic model) of immunocompromised mice, as described previously (58, 59). We observed a significant reduction of OC progression in the orthotopic mouse model treated with CP in ENPL KD cells transplanted mice compared to TR-127 WT cells treated with CP (2mg/kg) for 5 weeks (**Fig.1**). Another model included intraperitoneal (IP) injection of TR-127 cells into nude mice. These mice had significantly greater tumor growth and more numerous metastatic nodules in the mesentery, diaphragm, and pelvis than mice injected with TR-127 cells that had been treated with ENPL or exosome inhibitor either alone or combination with CP. When tumors were evident (based on MRI) the mice were assigned to one of five treatment groups: CP (Weekly), ENPL (Weekly). Tumor nodules were significantly reduced in the ENPL pre-treatment + CP group, compared to those treated with CP alone (**Fig.1A-C**), without affecting body weight, we have observed the significant reduction of tumor metastasis nodules in ENPL inhibitor combination with CP treatment than single treatment or without treatment (**Fig.1D**). Collectively, these data demonstrate that ENPL inhibitors, increased CP accumulation and specifically cytotoxic toward OC cells, resulting decreased tumor growth. This study is ongoing, we are currently evaluating ENPL and its target protein expression in mice tissues.

2. ENPL expression is highly elevated in chemoresistant OC. Membrane proteins are amongst the first members to sense any change in the event of pathological conditions. This easy accessibility renders them as perfect candidates for potential disease biomarkers with both diagnostic and prognostic potential. ENPL is one such membrane protein whose upregulation is associated with platinum resistance. Our *working hypothesis* is that the presence of ENPL in serum exosomes can serve as biomarkers for this resistance. To determine whether ENPL is most strongly associated with chemoresistance, we will evaluate the differential expression of ENPL in serum samples derived from OC patients using an ELISA in human OC serum samples were performed which revealed highly elevated ENPL expression in the chemo-resistant OC patient samples but absent or lower ENPL expression in the chemosensitive and control serum samples (**Fig. 2**). ENPL expression levels are 8-25 fold higher in chemo-resistant OC serum samples when compared with sensitive samples. This indicates that

ENPL expression is very specific to OC chemoresistant tissues and serum compared with benign or chemosensitive tissues and serum. We further validated this expression in a different set of OC platinum sensitive and resistant patients samples and demonstrated a high expression of ENPL in platinum sensitive than resistant samples when analyzed using ELISA (**Fig. 2B-D**). This supports its potential as a biomarker as well as a therapeutic target. Further, we have identified that combination of ENPL with CA125 showed increased sensitivity and specificity over individual expression in platinum-resistant serum EVs (AUC 0.93) **Fig. 2E**. In future study will validate these findings using large cohort of samples.

3. **Characterization of ENPL inhibition with L-2663 and L-2797 by exosome secretory pathway proteins and platinum accumulation.** Recently, we have identified that DAP compounds effectively suppress the membrane proteins such as TMEM205 and ENPL**.** To better understand the potential mechanism of action of DAP compounds L-2663 and L-2797 and how they affect exosome secretory pathways, several proteins were evaluated on WB analysis after treatment with each compound. Expression of exosome secretory pathway protein, Rab11, was significantly increased in PR cells (TR127 and PEOC4) after treatment with L-2663 (**Figure 3A & B**), while L-2797 decreased the expression of Rab11 in both cell lines. L-2663 treatment increased Rab27a in platinum resistant TR127 cells, but decreased Rab27a expression in other PR cells (PEOC4). These results suggest slightly different downstream effects of the two different DAP compounds as well as differential response in different cell lines which warrants further evaluation.

Exosome secretion was assessed by NTA in TR127 cells after treatment with 10 µM carboplatin at varying durations with increased secretion observed after 48 hours of treatment (**Figure 3C & D***).* Exosome secretion from PEOC4 cells also increased significantly after treatment with 10 µM carboplatin x24 hours (*Sup. Fig. 5A-B*). Cisplatin concentration was quantified in cell lysates as well as exosomes by collecting cells and isolating exosomes from conditioned culture media for platinum quantification by ICP-MS analyses. Prior to collection, PR TR127 cells were treated with either 10µM cisplatin for 6 hours or pre-treatment with L-2663 or AME (exosome inhibitor) prior to cisplatin treatment for 6 hours. These results demonstrated a significantly increased accumulation of cisplatin in TR127 cell lysates and a decreased exosomal concentration of cisplatin, in cells pre-treated with L-2663 and AME relative to the cisplatin treated control (**Figure 3E**). These findings were confirmed on ICC which demonstrated that intracellular/nuclear accumulation of Texas-red labeled cisplatin was increased in TR127 and PEOC4 cells pre-treated with L-2663 (*Figure 4F***).** Collectively, these data indicate that TMEM205-mediated exosome secretion plays a key role in platinum-resistance by modulating platinum efflux.



ENPL inhibitor increases CP sensitivity in OC peritoneal mice. A TR-172 cells (10^{-5}) injected i.p and 50uL of purified from normoxic and hypoxic cultured cells exosomes containing ENPL (100 µg) were i.p. injected into mice twice a week for 3 weeks. The exosome treatments were started on day 3 after tumor cell injection so that sufficient time was given for the attachment of the cancer cells to the inner side of the abdominal wall and the surfaces of the intraperitoneal organs. Aggressive metastasis growth and the number of metastatic nodules was significantly higher (circle indicated) in mice injected with hypxic exosomes containing ENPL. **B**) Mice treated with ENPL inhibitor (5mg/kg, i.p), CP (2mg/kg, i.p), ENPL inhibitor in combination with CP showed significant anti-tumor efficacy compared to CP treatment alone. (Study is ongoing to complete the ENPL and its target protein expression in mice tissues)

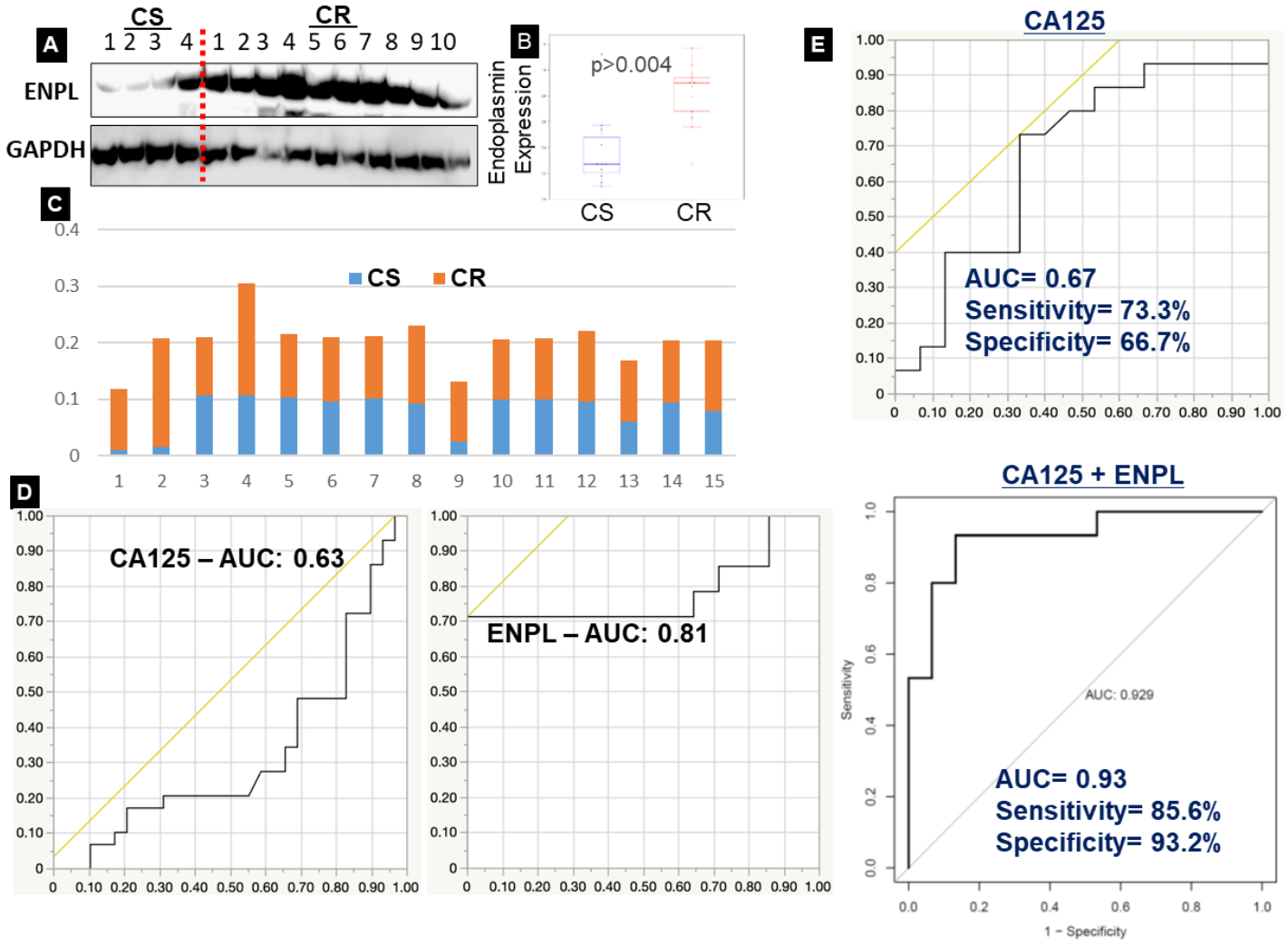


Figure 2. ENPL expression in OC patients: A & B) ENPL expression is highly elevated in OC platinum resistant patient serum or tissues than platinum sensitive (n=10 for WB; n=4 for IHC); **C)** High ENPL in OC patient serum samples than OC platinum sensitive serum exosomes samples (n-18); **D)** ROC curves of CA125, and ENPL based on ELISA results from exosomes isolated from platinum sensitive and resistant disease of OC serum samples. ENPL had an AUC of greater than 0.81, compared to CA125 0.63 (aka MUC16) analyzed (n=16). **E**). Our candidate protein ENPL expression combination with CA125 had an AUC of greater sensitivity specificity AUC0.93, compared to CA125 0.67 or ENPL 0.81 (n=15).

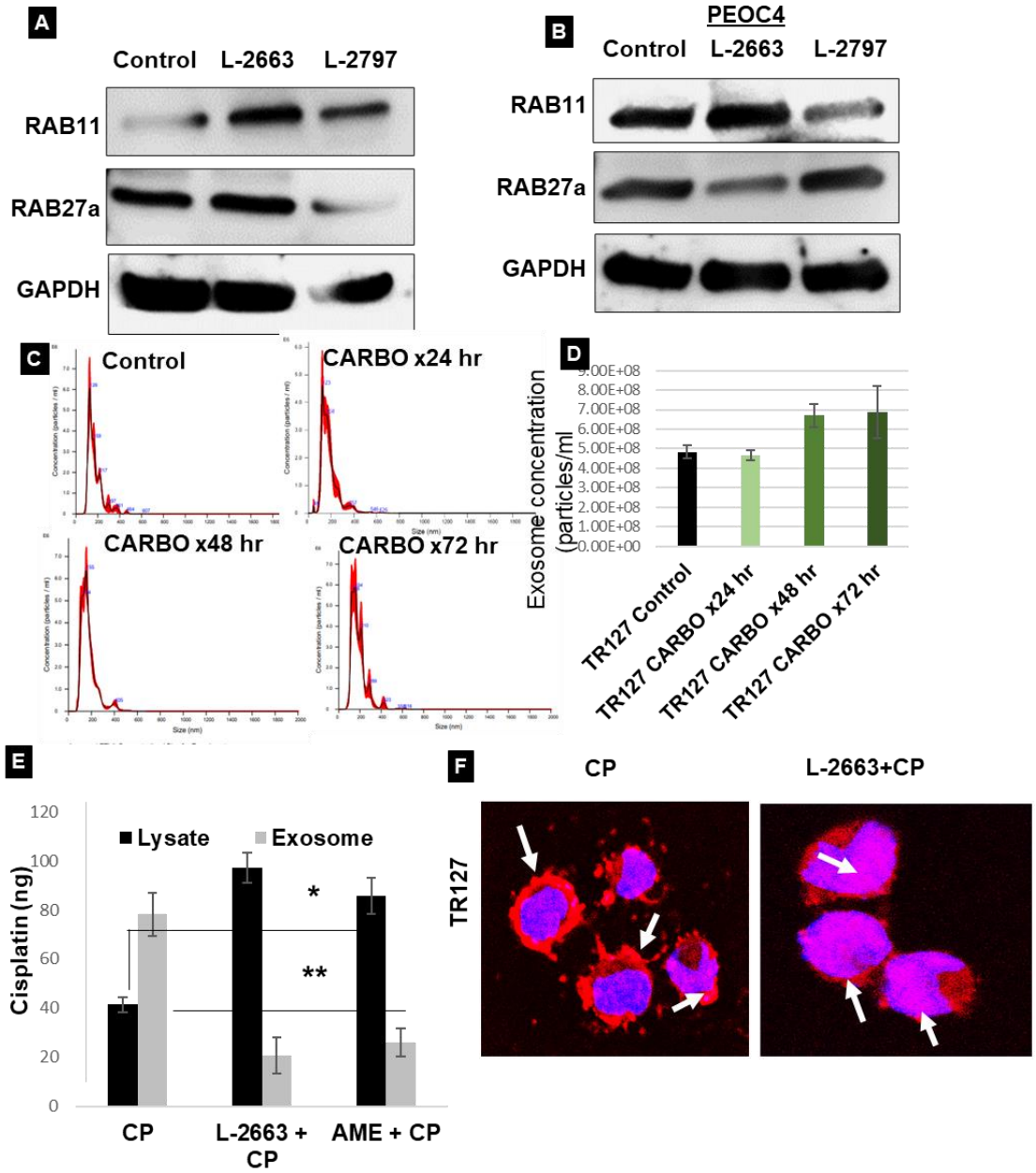


Figure 3. Effects of ENPL inhibition on exosomal secretory pathway proteins, increased exosome efflux with platinum treatment, and increased intracellular accumulation of platinum with TMEM205 inhibition A & B) TR127 and PEOC4 cells treated with 10 µM L-2663 or L-2797. L-2663 treatment was significantly increased Rab11in both PR cells, Rab27a increased in TR127 cells and decreased in PEOC4 cells in L-2663 treatment. L-2797 treatment was decreased Rab11 in both cells for 24 h. **C & D**) Nanoparticle tracking analysis (NTA) to determine exosome concentration in TR=127 cells treated with carboplatin at different time points, increased exosome secretion levels were observed after 48 hours (n=5, $p<0.05$). **E**) ICP-MS analysis of cisplatin accumulation in cell lysates and exosomes of TR127 cells treated with cisplatin alone, versus pre-treatment with L-2663 + cisplatin or pre-treatment with AME + cisplatin for 24h (n=5, $p\leq0.005$). **F**) Immunofluorescence of TR127 and PEOC4 cells treated with GFP labeled CP or TexasRed-labeled CP. CP is localized on the outer membranes of the cells (green or red) whereas L-2663 pre-treated cells show CP accumulation in the nuclei.

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?

Currently we are finalizing the manuscript describing the data presented in the report. Plan to submit this final study works to Gynecology Oncology in Feb 2023.

4. IMPACT

1. Impact on the development of the principal discipline (ovarian cancer) of the project: Identifying the role of endoplasmin in the development of platinum-resistance of OC: Our preliminary results showing that ENPL could play a role in in promoting OC progression and chemoresistance through the exosome secretion payways. Based on these findings, we will evaluate novel mechanisms linking ENPL with exosomal secretion, including, how ENPL expression plays a role in exosome release and contributes to OC chemoresistance through the MRP2 activation in OC in SA1 and SA2. This finding will potentially lead to the identification of novel biomarkers and therapeutic targets for chemoresistant OC.

2. Impact on the development of other disciplines: Our study can have impact on all other solid tumors. By identifying the role of ENPL and combining the approaches of blocking exosome secretion with cisplatin treatment approaches, the outcome of combination thearpy can me enhanced and made for successful for the patient.

3. Impact of the technology transfer: Translational Technology - Microfluidics device: We have developed a novel microfluidics based device to isolate intact exosomes with greater purity and quality in a shorter time that will allow for downstream processing. These factors are critical for moving forward in clinical translation and be directly applicable for exosome-based biomarker screening in patient serum samples.

4. Impact on society beyond science and technology: nothing to report.

5. CHANGES & PROBLEMS

Changes: Nothing to report

Problems: We faced a problem with our ENPL antibody issue in patient serum sample study by ELISA and few research supplies were delay due to COVID. This significantly delayed our in vivo study experiments. We solved the problems within extended period of this projects, purchased new antibody in different company, worked well in patient samples serum and tissue.

- **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

6. PRODUCTS

- **Publications, conference papers, and presentations**
  Role of ENPL mediated platinum resistant in ovarian cancer. Dorayappan KDP, Saini U, Colin H, Takahiko S, Smith BQ, Lightfood M, Flannery M, Hays J, Hansford D, O'Malley D, Cohn DE, **Selvendiran K**. *Manuscript under preparation for Gynecology Oncology 2023.*

- **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**
  We have developed a novel microfluidics based device to isolate intact exosomes with greater purity and quality in a shorter time that will allow for downstream processing. These factors are critical for moving forward in clinical translation and be directly applicable for exosome-based biomarker screening in patient serum samples.

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project:

Name: Selvendiran Karuppaiyah
Project Role: PI
No Change

Name: David Cohn
Project Role: Co-I
No Change

Name: Jing Zhao
Project Role: Biostatistician
No Change

Name: Kalpana Deepa Priya Dorayappan
Project Role: Post Doc Fellow
No Change

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

Active Support Changes:

Selvendiran Karuppaiyah (PI)

Now Active / Awarded:
1. DOD FY20 Ovarian Cancer Research Program - Clinical Translational Research Award
W81XWH2110427 06/15/2021 – 06/14/2023 3 calendar months

2. DOD FY21 Ovarian Cancer Research Program - Clinical Translational Research Award

W81XWH-22-1-0371 06/15/2022 – 06/14/2024 3 calendar months.

3. DOD FY21 Peer Reviewed Cancer Research Program IMPACT Award

W81XWH-22-1-0656 06/15/2022 – 06/14/2026 4 calendar months.

Jing Zhao (Biostatistician)

Active / Awarded:
DOD FY20 Ovarian Cancer Research Program - Clinical Translational Research Award W81XWH2110427 Total Costs: 06/15/2021 – 06/14/2023 0.6 calendar months

Active / Awarded:
Role: **Biostatistician**
Nat In. Arthritis & Muscoskeletal & Skin
Title: Skeletal muscle in rheumatoid arthritis

K23AR068450 Total Costs: 09/012020 – 08/31/2021 2.4 calendar months

Active / Awarded:
Role: Biostatistician
National Institute of Neurological Disorders and Stroke
Title: Reducing infection susceptibility by immune function restoration in spinal cord injury
R01NS118200 Total Costs: 07/01/2020 – 06/30/2022 0.6 calendar months

Active / Awarded:
Role: Biostatistician
National Institute of Neurological Disorders and Stroke
Title: Implementation of machine learning workflows in primary brain tumor diagnostics R03NS116334 Total Costs: 06/01/2020 – 11/30/2021 0.6 calendar months

Active / Awarded:
Role: Biostatistician
NCI
Title: The translational regulation of pro-apoptotic genes
R01CA251753 Total Costs: 07/14/2020 – 06/30/2025 1.2 calendar months

Active / Awarded:
Role: Biostatistician
National Heart, Lung and Blood Institute
Title: ISGylation regulates lung endothelial inflammation
R01HL157164 Total Costs: 04/20/2021 – 03/31/2025 1.2 calendar months

Active / Awarded:
Role: Biostatistician
NCI
Title: Validating urine derived cancer cells (UDCC) – non-invasive and living liquid biopsies – in bladder cancer clinics
R33CA258016 Total Costs: 05/01/2021 – 04/30/2024 0.60 calendar months

What other organizations were involved as partners:

1. Additional OC platinum resistant and sensitive serum samples was provided by **Dr. Larry Maxwell, MD** at **Inova Schar Cancer Center** for evaluate the clinical significance of ENPL expression as a marker of platinum resistance and survival in OC patient samples.

Nothing to report on any other personnel's and relationships.

8. Special Reporting Requirements

Nothing to report

9. APPENDICES