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14. ABSTRACT
The application of HT-RNAi represents an innovative functional genomic strategy to rapidly identify important genes involved in the response of cancer cells to the chemotherapeutic agents. We developed a novel HT-RNAi screening assay using a kinase siRNA library to identify genes that modulate the response of ovarian cancer cells to cisplatin and paclitaxel. HT-RNAi assays were conducted using the cell line SKOV-3 to identify genes that sensitize cells to low dose cisplatin. Similar screening assays were done using low dose paclitaxel. Analysis of the screening data resulted in lists of sensitizing hits. Validation of the genes identified as sensitizers was initiated and we confirmed the kinases CHK1 and ATR as sensitizing targets to cisplatin. Validation of the genes that sensitize to paclitaxel is currently underway. Further confirmation of gene silencing by the functional siRNA also showed that CHK1 is being significantly silenced by siRNA targeting CHK1 and further silencing confirmation is in progress. Identification and confirmation of kinases involved in modulating the response to cisplatin and paclitaxel will provide the basis for the development of more effective therapeutic strategies in the treatment of ovarian tumors.

15. SUBJECT TERMS (key words)
RNAi, High-throughput RNAi, SKOV-3, cisplatin, paclitaxel

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INTRODUCTION:

Ovarian cancer, the most aggressive gynecologic cancer, is the foremost cause of death from gynecologic malignancies in the developed world. Two primary reasons explain its aggressive behavior: most patients present with advanced disease at diagnosis, and die of recurrences from disease that has become resistant to conventional chemotherapies. According to the 2007 American Cancer Society statistics, nearly 55%, or 15,280 of the 28,020 expected deaths from gynecologic malignancies, are be attributed to ovarian cancer (Jemal et al., 2007). Despite aggressive primary therapy and high initial response rates (RRs), most women with advanced ovarian carcinoma will relapse and develop drug-resistant disease (du Bois et al., 2003a; du Bois et al., 2003b). Following initial surgery, the large majority of patients with this malignancy will receive a chemotherapy regimen that includes a platinum drug (carboplatin or cisplatin) and a taxane (paclitaxel or docetaxel). Unfortunately, even in patients in whom there is an initial positive clinical response, the development of recurrent drug-resistant ovarian tumors is a common outcome, leading to poor 5-year survival rates of below 30%. The development of a more effective chemotherapy treatment by identifying genes involved in modulating drug responses would be instrumental in the ability to fight this disease. To address this issue, we developed an innovative functional pharmacogenomics approach based on high-throughput RNA interference (HT-RNAi) analysis (Dorsett and Tuschl, 2004). We propose to utilize a focused siRNA (short-interfering RNA) library targeting the human 'kinome' to identify potentiators of cisplatin and paclitaxel response that control ovarian cancer cell proliferation and survival. The identification of kinase-mediators that enhance the chemotherapeutic response will serve as a starting point to the development of new therapeutic targets and lead to a more thorough understanding of chemotherapeutic response of cisplatin and paclitaxel in ovarian cancer. The overall aim of this study is to apply an innovative functional genomic strategy to identify genes that act as sensitizers of cisplatin and/or paclitaxel response in ovarian cancer cells.

BODY:

Specific Aim 1: *To determine the effect of targeted reduction of 572 kinases on growth of OVCAR-3 and SKOV-3 ovarian cancer cells to cisplatin and paclitaxel.*

In order to examine the effects of cisplatin and paclitaxel on ovarian cancer cell growth, we first began by selecting a cell line model that would be applicable to HT-RNAi. We originally chose OVCAR3 and SKOV-3 ovarian cancer cell lines for these experiments, but we also tested two other cell lines A2780 and A2870cis, both of which had differential sensitivity to cisplatin. All the cell lines were commercially available and are described in Figure 1. Assay development for HT-RNAi conditions was initiated on all the four cell lines. Three out of the four cell lines, SKOV-3, A2780 and A2780cis grew at a good rate and

A

Cell Line	Source	Cat. #	Description
SKOV-3	ATCC	HTB-77	Human Ovarian Adenocarcinoma
OVCAR-3	ATCC	HTB-161	Human Ovarian Epithelial Adenocarcinoma
A2780	ECACC	93112519	Human Ovarian Carcinoma
A2780cis	ECACC	93112517	Human Ovarian Carcinoma - cisplatin resistant

B

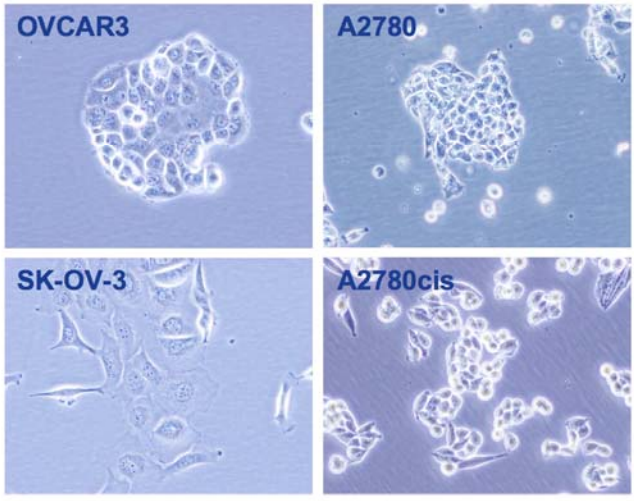

Figure 1. **Human ovarian cancer cell lines. (A)** Table shows the ovarian cancer cell lines used in this project. **(B)** Bright-field images of human ovarian cancer cells, Magnification_x20.

were candidates for further assay development.

HT-RNAi Assay Development

Assay development consisted of assaying the cell lines for two parameters needed for HT-RNAi. The first is the selection of the optimal transfection reagent for each cell line. For this, the cell lines were transfected with negative control (non-silencing) siRNA, positive control (lethal) siRNA and no siRNA, using various concentration of eight commercially available, cationic lipid transfection reagents. These assays were performed using the 384-well plate format, the desired cell number and over the optimal growth period to maximize siRNA transfection. For the three cell lines used, we chose to seed 1000 cells per well and grow them for 96 hours. From these transfection reagent optimization studies, siLentFect at a 5:1 (vol:wt) ratio was identified as being optimal for all the three cell lines (Data not shown).

The second parameter needed for the HT-RNAi screen was the selection of the drug concentrations of both cisplatin and paclitaxel for screening. These drug dose response (DDR) assays were also performed in 384-well plates, at a seeding density of 1000 cells per well and a 72-hour drug exposure totaling to 96 hour assay duration. The read out for these experiments was the cell proliferation assay Cell Titer Glo (Promega) and this readout was used in all the subsequent HT-RNAi screens. The DDR assays were seeded at the desired cell concentration and at 24 hours, the cells were treated with varying concentrations of either cisplatin or paclitaxel. After 72 hours of drug exposure, cell number was obtained using Cell Titer Glo. Raw relative luminescent units (RLU) were compared to untreated control wells for normalization and plotted (Figure 2). Results indicate that the three cell lines have variable responses to cisplatin and paclitaxel. IC50 values for cisplatin were 2 µM for SKOV-3, 850 nM for A2780 and 6.5 µM for A2780cis, respectively. IC50 values for paclitaxel were 12 nM for SKOV-3, 1 nM for A2780 and 1.9 nM for A2780cis. Further DDR assays were done using straight dilutions of drug in order to select the concentration needed to give an IC10-IC30 response for each drug (data not shown).

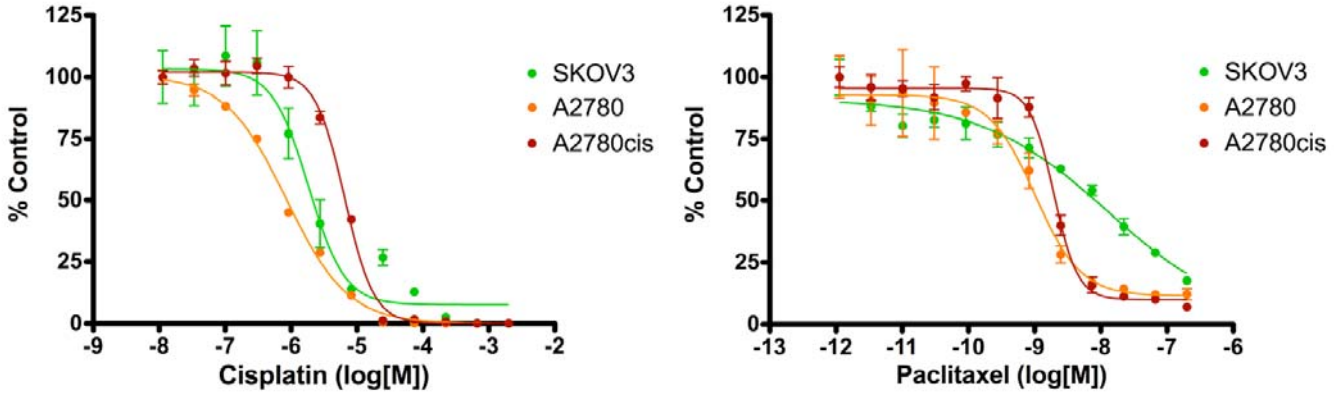

Figure 2. Drug dose response of ovarian cancer cell lines to cisplatin and paclitaxel. SKOV-3 cells were seeded onto 384-well plates and at 24 hours cells were treated with varying concentrations of either cisplatin (Left graph) or paclitaxel (Right graph). Cells were allowed to grow for an additional 72 hours. Plates were assayed for cell number using Cell Titer Glo (Promega). Values were normalized to untreated control wells and plotted using Prism (Graphpad).

HT-RNAi Assay Validation Screen

Once the optimal transfection reagent and drug concentrations was identified, we conducted small-scale screens under the established assay conditions. The siRNA set used was an in-house siRNA library targeting 334 genes (818 siRNAs) that contained control siRNAs. Assay plates were prepared by printing 1-2 µl of diluted siRNA onto white 384-well tissue culture plates. Printed plates were sealed and stored at -80˚C until they were ready to be used. SKOV-3 cells were transfected with validation set siRNA using reverse transfection method (Aza-Blanc et al., 2003). Replicate sets were prepared and a schematic diagram of the assay is shown in Figure 3. At 24 hours, IC10-30 dose of either cisplatin or paclitaxel was added to one set and vehicle was added to the other set. After 72 hours, both sets were treated with Cell Titer Glo and assayed for luminescence. RLU were measured and normalized to control. Validation screens were performed for SKOV-3 and A2780 cells. Since the A2780 cells showed growth edge-effects, an artifact of 384-well plate based screening, we decided to initiate all screens using SKOV-3 cells. The target concentrations for SKOV-3 cells were 2 µM (IC20) for cisplatin and 10 nM (IC30) for paclitaxel. Next, we proceeded with HT-RNAi using the kinase siRNA set.

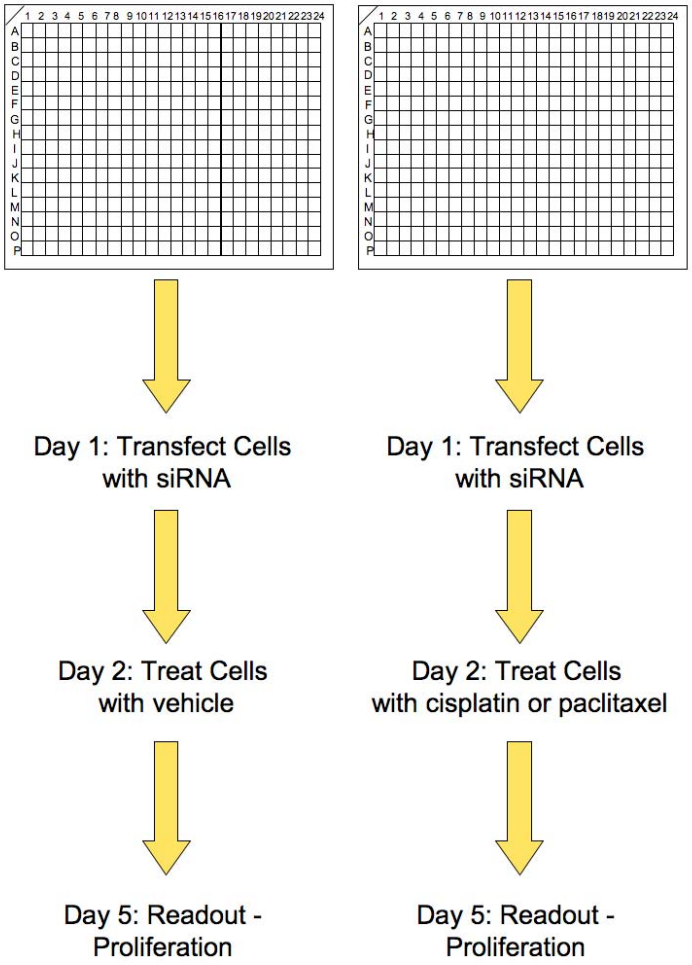

Figure 3. Screening assay for modulators of cisplatin or paclitaxel response. Schematic Diagram of HT-RNAi assay. Cells are transfected with siRNA in 384-well plates, treated with vehicle, cisplatin or paclitaxel at 24 hours. Seventy-two hours later, plates are analyzed for growth modulation activity.

HT-RNAi Kinase Screen

We next performed a HT-RNAi screen using the validated kinase siRNA library (Qiagen), which contains 1144 siRNA targeting 572 kinase genes (2 siRNA/gene). The kinase siRNA set was printed onto assay plates as described above. The assay was performed identically to the assay validation screen with one adjustment of seeding 800 cells/well instead of 1000 cells/well. The assay was also repeated to provide a biological replicate. Data generated from these duplicate screens was analyzed using several data analysis techniques used in our laboratory to generate a "hit list" of kinases that modulate the response of SKOV-3 cells to either cisplatin or paclitaxel. One method of analysis normalized the values to the untreated controls. The plot of values analyzed in this way for both kinase siRNA screens treated with cisplatin or vehicle is shown in Figure 4. Each dot represents the growth effect of a specific kinase siRNA treated with either vehicle or cisplatin. As can be seen, most siRNAs do not modulate the response of the cells to cisplatin, but there are several data points that are outside the diagonal showing very strong potentiation of the cisplatin effect. We highlighted two genes CHK1 and ATR where both siRNA sequences had similar effects on sensitizing SKOV-3 cells to cisplatin.

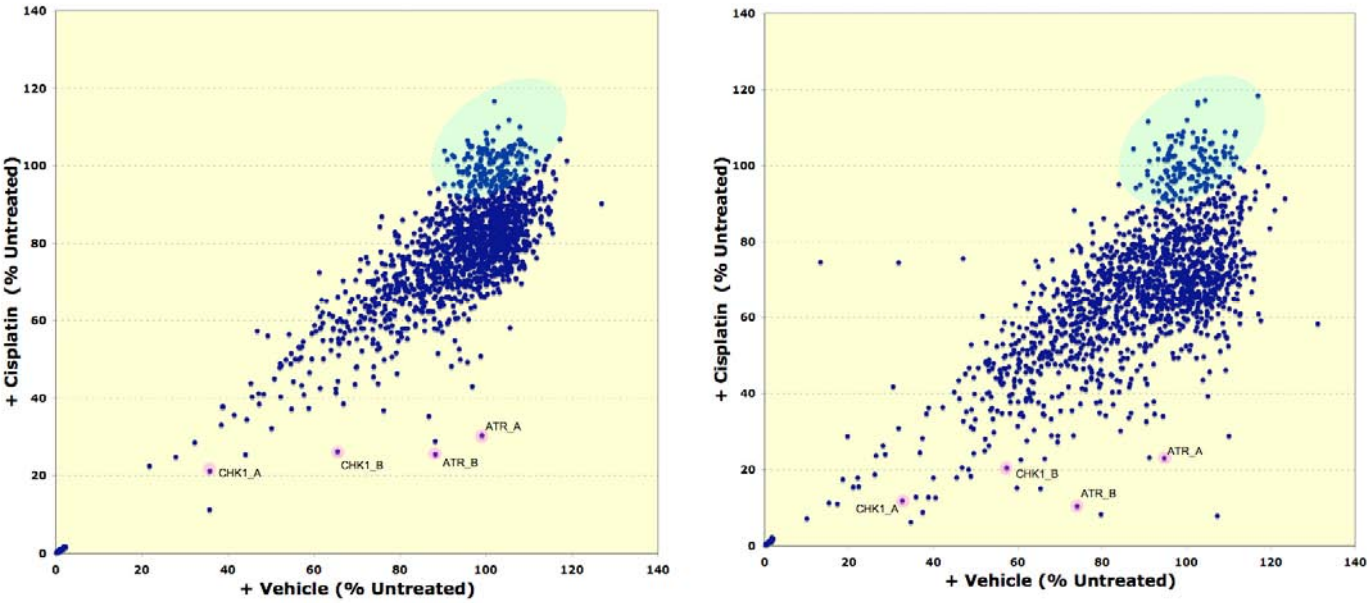

Figure 4. Screening a kinase siRNA library for modulators of cisplatin activity. SKOV-3 cells were transfected with siRNA library in duplicate plates and 24 hours later treated with 2 µM cisplatin. Cells were allowed to grow an additional 72 hours. Plates were assayed for cell number using Cell Titer Glo (Promega). Values were normalized to control wells and plotted as vehicle/siRNA treated vs cisplatin/siRNA treated. Biological replicates were run for the screen (left and right graphs). The siRNA showing potentiation of cisplatin activity are highlighted in pink and labeled. Reference untreated wells are highlighted in the blue shaded area.

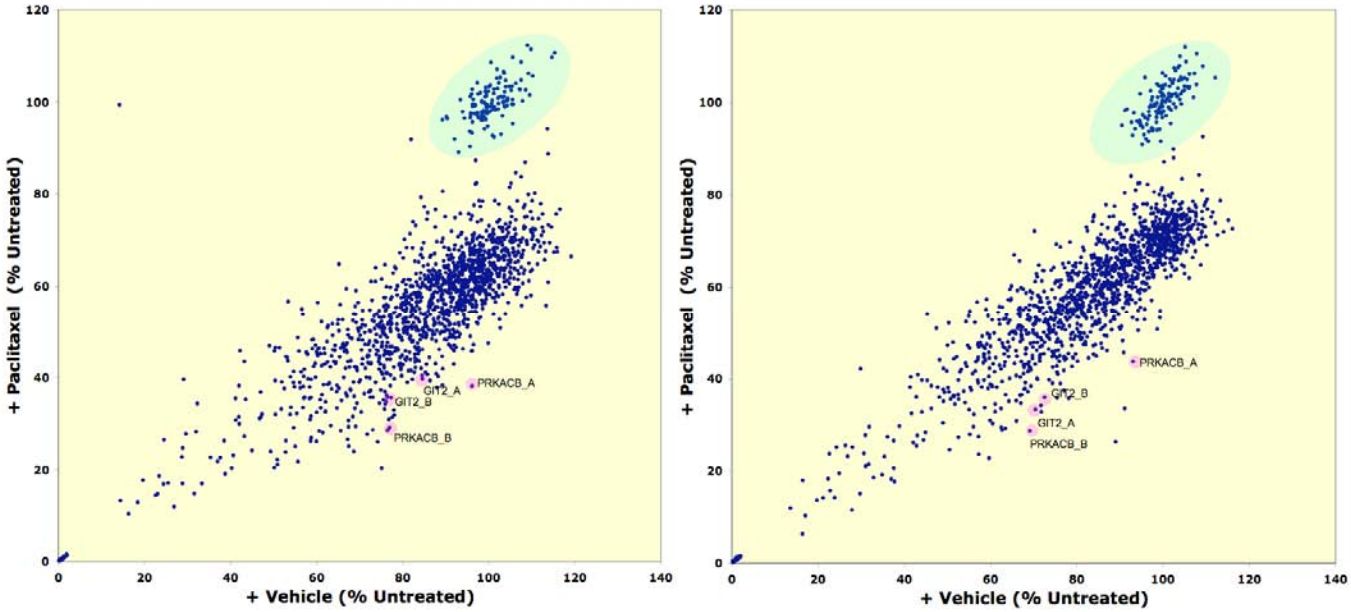

Figure 5. Screening a kinase siRNA library for modulators of paclitaxel activity. SKOV-3 cells were transfected with siRNA library in duplicate plates and 24 hours later treated with 10 nM paclitaxel. Cells were allowed to grow for an additional 72 hours. Plates were assayed for cell number using Cell Titer Glo (Promega). Values were normalized to control wells and plotted as vehicle/siRNA treated vs cisplatin/siRNA treated. Biological replicates were run for the screen (left and right graphs) and siRNA showing potentiation of paclitaxel activity are highlighted in pink and labeled. Reference untreated wells are highlighted in the blue shaded area.

Similarly, we performed HT-RNAi screens on SKOV-3 cells and treated with 10 nM (IC30) paclitaxel. These screens were also run in duplicates. Results of these HT-RNAi kinase screens are shown in Figure 5. Two genes that showed slight potentiation and where both siRNA showed similar effects were GIT2 and PRKACB. Further validation of all the gene hits is required to show true potentiation of drug effect.

Specific Aim 2*: To validate the kinase gene knockdowns that sensitize OVCAR-3 and SKOV-3 cells to cisplatin and paclitaxel.*

In specific aim two, we proposed to select the siRNA targeting genes from the hit lists and evaluate them for further confirmation. Since we performed biological replicate runs for each screen, a functional confirmation screen was not needed to confirm the screening results. Instead, we prioritized siRNA hits that gave similar effects in both screens and where both siRNA sequences showed similar results. Functional validation was done using siRNA targeting the hits and treating with varying doses of drug. For functional validation of sensitizing activity of CHK1 and ATR siRNA plus cisplatin in SKOV-3 cells, we performed a siRNA-DDR assay, which silenced the target genes CHK1 or ATR and measured the response of SKOV-3 to varying doses of cisplatin (Figure 6). We are currently in the process of further functional validation of several gene targets for both cisplatin

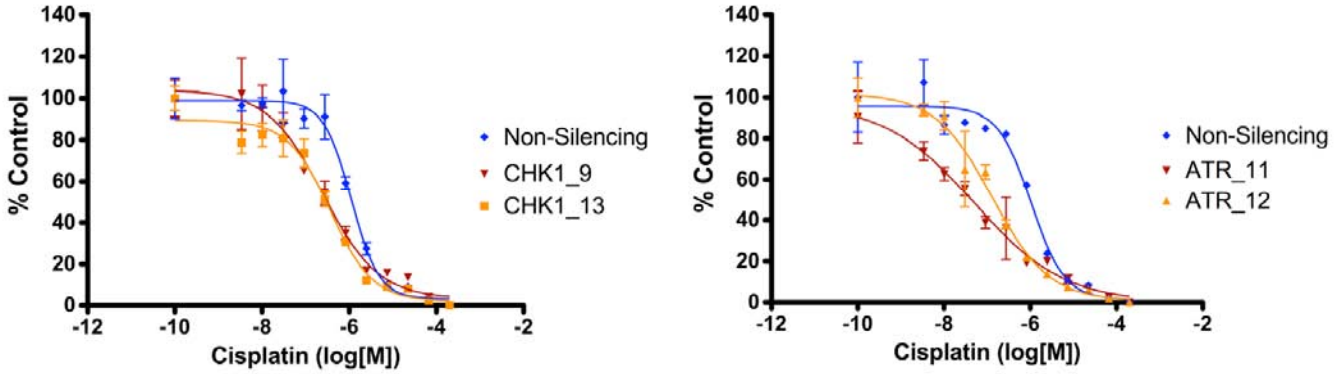

Figure 6. Effect of CHK1 and ATR silencing on drug dose response of SKOV-3 ovarian cell lines to. SKOV-3 cells were transfected with non-silencing siRNA or siRNA targeting CHK1 (Left graph) or ATR (Right graph) by reverse transfection on 384-well plates and at 24 hours cells were treated with varying concentrations of cisplatin. Post drug addition, cells were allowed to grow for an additional 72 hours.. Plates were assayed for cell number using Cell Titer Glo (Promega). Values were normalized to untreated control wells and plotted using Prism (Graphpad).

and paclitaxel. As part of functional validation, we performed similar siRNA-DDR assays on two other ovarian cancer cell lines A2780 and another cell line that we found transfects well with siRNA, CaOV3. Results indicate a slight sensitization effect of ATR and CHK1 siRNAs in CaOV3 and less of an effect in A2780 (Data not shown). Repeats of these assays and further functional validation are in progress.

We proposed to validate specific siRNA mediated gene silencing by using either western blot analysis and/or Q-PCR (quantitative-PCR) assays to show decreased expression of specific kinases. We have used western blot analysis to validate the gene silencing of four of the siRNA targeting CHK1. Results show that the four siRNA did markedly decrease the amount of CHK1 in the SKOV-3 cells (Figure 7). Similar experiments will be done to validate the gene silencing of targets that sensitize cells to cisplatin and paclitaxel.

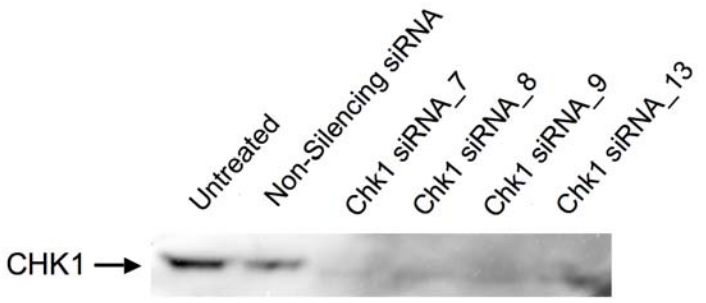

Figure 7. **Confirmation of gene silencing by CHK1 siRNA.** SKOV-3 cells were transfected with four different CHK1 siRNA or controls and allowed to grow for 72 hrs. Cells were lysed and 30 µg of lysate from each treatment was analyzed by western blot for expression of CHK1. A rabbit anti-CHK1 Ab was used for detection.

Further characterization of the role of these kinases in response to cisplatin and paclitaxel in ovarian cancer cells will also be performed. To kinase targets where specific inhibitors already exist, we will use these inhibitors and test them in combination with cisplatin or paclitaxel for their ability to produce a synergistic effect. We anticipate validation of the gene hits to be completed in approximately six to eight weeks. Furthermore, we plan to include a cisplatin-resistant cell line A2780cis and a paclitaxel resistant cell line A2780pacli55, which was developed as part of this project, in future validation experiments. These results will be the basis for planning future *in vivo* studies using xenograft models, which is beyond the scope of the current proposal.

KEY RESEARCH ACCOMPLISHMENTS:

- Optimized siRNA transfection conditions for the SKOV-3 ovarian cancer cell line for HT-RNAi screening.
- Optimized siRNA transfection conditions for the A2780 ovarian cancer cell line for HT-RNAi screening.
- Established a HT-RNAi screening assay for the identification of sensitizing targets of cisplatin and paclitaxel in SKOV-3 cells.
- Performed a kinase siRNA library screening for modulators of cisplatin response.
- Performed a kinase siRNA library screening for modulators of paclitaxel response.
- Identified kinase genes whose silencing enhanced the response of SKOV-3 cells to cisplatin.
- Identified kinase genes whose silencing enhanced the response of SKOV-3 cells to paclitaxel.
- Validated CHK1 and ATR as sensitizers of cisplatin response in SKOV-3 cells.
- Optimized siRNA transfection conditions for the CaOV3 ovarian cancer cell line for validation.
- Validated CHK1 and ATR as sensitizers of cisplatin response in CaOV3 cells.
- Selected genes for further validation experiments.
- Developed a paclitaxel resistance A2780 cell line.

REPORTABLE OUTCOMES:

- Peralta, L.A., Basu, G.D., Choudhary, A., Monzon, I.G., and Azorsa, D.O. High-throughput RNAi phenotypic screening for the identification of genes that cisplatin response in human ovarian cancer cells. *American Association for Cancer Research Annual Meeting 2008. San Diego, CA. April 12-16, 2008* (Poster presentation), published in AACR annual meeting proceedings, 49 (Cellular and molecular biology 13): 215.

- Arora, S., Peralta, L.A., Bisanz, K.M., Choudhary, A., and Azorsa, D.O. Synthetic lethal RNAi screening for the identification of genes that modulate cisplatin and paclitaxel response in human ovarian cancer cells. *American Association for Cancer Research Annual Meeting 2009. Denver, CO. April 18-22, 2009* (submitted for presentation)

- Arora, S., Peralta, L.A., Bisanz, K.M., Gonzales, I.M., Choudhary, A., Basu, G.D., and Azorsa, D.O. Synthetic lethal RNAi screening for the identification of genes that modulate cisplatin and paclitaxel response in human ovarian cancer cells. (Manuscript in preparation).

- Cell line developed: Paclitaxel-resistant A2780 cell line designated A2780pacli55.

CONCLUSION:

The application of HT-RNAi represents an innovative functional genomic strategy to rapidly identify important genes involved in the response of ovarian cancer cells to the chemotherapeutic agents cisplatin and paclitaxel. We developed a novel HT-RNAi screening assay using an siRNA library targeting 572 kinases to identify genes that modulate the response of ovarian cancer cells to cisplatin and paclitaxel. HT-RNAi assays were conducted using the ovarian cancer cell line SKOV-3 to identify genes that sensitize cells to low dose cisplatin. Similar screening assays were done using low dose paclitaxel. Analysis of the screening data resulted in lists of sensitizing hits. Validation of the genes identified as sensitizers was initiated and we confirmed the kinases CHK1 and ATR as sensitizing targets to cisplatin. Validation of the genes that sensitize to paclitaxel is currently underway. Further confirmation of gene silencing by the functional siRNA also showed that CHK1 is being significantly silenced by siRNA targeting CHK1 and further silencing confirmation is in progress. Identification and confirmation of kinases involved in modulating the response to cisplatin and paclitaxel will provide the basis for the development of more effective therapeutic strategies in the treatment of ovarian tumors.

REFERENCES:

Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL and Cooke MP (2003) Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Molecular cell* **12**:627-637.

Dorsett Y and Tuschl T (2004) siRNAs: applications in functional genomics and potential as therapeutics. *Nature reviews* **3**:318-329.

du Bois A, Luck HJ, Meier W, Adams HP, Mobus V, Costa S, Bauknecht T, Richter B, Warm M, Schroder W, Olbricht S, Nitz U, Jackisch C, Emons G, Wagner U, Kuhn W and Pfisterer J (2003a) A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. *Journal of the National Cancer Institute* **95**:1320-1329.

du Bois A, Pfisterer J, Meier W and Wagner U (2003b) Improving first-line therapy of advanced ovarian cancer - the AGO Ovarian Cancer Study Group perspective. *Int J Gynecol Cancer* **13 Suppl 2**:169-171.

Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ (2007) Cancer statistics, 2007. *CA: A cancer journal for clinicians* **57**:43-66.

APPENDICES:

None

SUPPORTING DATA:

None