Small Molecules Identified from a Quantitative Drug Combinational Screen Resensitize Cisplatin’s Response in Drug-Resistant Ovarian Cancer Cells

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Abstract

Drug resistance to chemotherapy occurs in many ovarian cancer patients resulting in failure of treatment. Exploration of drug resistance mechanisms and identification of new therapeutics that overcome the drug resistance can improve patient prognosis. Following a quantitative combination screen of 6060 approved drugs and bioactive compounds in a cisplatin-resistant A2780-cis ovarian cancer cell line, 38 active compounds with IC50s under 1 μM suppressed the growth of cisplatin-resistant ovarian cancer cells. Among these confirmed compounds, CUDC-101, OSU-03012, oligomycin A, VE-821, or Torin2 in a combination with cisplatin restored cisplatin’s apoptotic response in the A2780-cis cells, while SR-3306, GSK-923295, SNX-5422, AT-13387, and PF-05212384 directly suppressed the growth of A2780-cis cells. One of the mechanisms for overcoming cisplatin resistance in these cells is mediated by the inhibition of epidermal growth factor receptor (EGFR), though not all the EGFR inhibitors are equally active. The increased levels of total EGFR and phosphorylated-EGFR (p-EGFR) in the A2780-cis cells were reduced after the combined treatment of cisplatin with EGFR inhibitors. In addition, a knockdown of EGFR mRNA reduced cisplatin resistance in the A2780-cis cells. Therefore, the top active compounds identified in this work can be studied further as potential treatments for cisplatin-resistant ovarian cancer. The quantitative combinational screening approach is a useful method for identifying effective compounds and drug combinations against drug-resistant cancer cells.

Introduction

The majority of ovarian cancer patients are initially responsive to platinum- and paclitaxel-based chemotherapy [1]. However, over 60% of these patients relapse after a few cycles of chemotherapy [2]. For the patients with relapsed ovarian cancer, resistance to conventional chemotherapy develops in almost all cases. Addition of a third, broadly cytotoxic drug to the chemotherapy regimen has not been very successful [3,4]. The underlying mechanisms for resistance to platinum-based compounds are complex and still not well understood [5]. There is an urgent need to develop novel methods and approaches to bridge the translational gap between basic ovarian cancer research and clinical practice.

Next-generation sequencing studies have identified genes that are potentially responsible for drug resistance in cancer patients [6,7], and a drug repurposing screen of focused cancer drugs produced effective precision treatment leading to stabilized tumor size and longer survival [8]. In the past decade, a combination of cytotoxic drugs and...
vascular endothelial growth factor (VEGF)-targeted drugs, such as bevacizumab, has shown improved progression-free survival in Phase III trials [9,10]. These results indicate that targeted therapy may directly attack the specific mechanism of drug resistance and resensitize the cancer cells to cytotoxic agents, leading to a more effective precision treatment. A promising approach of combining genetic analyses and pharmacological screening of 76 target-specific compounds identified effective drug combinations in patient-derived, drug-resistant, non-small cell lung cancer models [11]. Although there has been some success in using focused drug collections for identifying combinational agents, a larger and more diverse drug collection could provide better opportunities to discover new active compounds to overcome specific drug resistance.

Using a drug-resistant ovarian cancer cell line, we screened three compound libraries: 2808 approved drugs from US, Canada, the UK, the EU, and Japan [12]; a focused collection of 1920 mechanism-based bioactive compounds with many protein kinase inhibitors and protease inhibitors [13]; and the Library of Pharmacologically Active Compounds (LOPAC). Several approved drugs and synergistic drug pairs were successfully identified from these compound collections in previous screens [14–17]. Here, we present a quantitative combinatorial screening approach for rapid identification of effective compounds, acting by themselves or in drug combinations, which suppressed the growth of cisplatin-resistant ovarian cancer cells. In addition to the single active compounds, EGFR inhibitors and several other compounds in combination with cisplatin resensitized drug-resistant ovarian cells to cisplatin. Restoration of overexpressed EGFR and increased p-EGFR levels by EGFR inhibitors were observed, and knockdown of EGFR expression also reduced the resistance to cisplatin in these cancer cells. These newly identified compounds could be studied further for the potential treatment of cisplatin-resistant ovarian cancer. Our results demonstrate that this quantitative drug combinational screening approach can identify effective new compounds against drug-resistant cancer cells, as well as useful two-drug combinations for resensitizing cancer cells to cisplatin.

**Results**

**Quantitative Combination Drug Repurposing Screen With a Cisplatin-Resistant Ovarian Cancer Cell Line**

A cell viability assay measuring cellular ATP content was developed and optimized to determine cisplatin’s response in the cisplatin-resistant A2780-cis cell line and its parent A2780 line (Figures 1, A and B, and S1, A–D). The cisplatin potency (a half maximal inhibitory concentration, IC₅₀) was 20.8-fold less potent in the A2780-cis cells (IC₅₀ = 13.4 μM) than in the sensitive A2780 cells (IC₅₀ = 0.65 μM) (Figure 1A). The A2780-cis cells were similarly resistant to carboplatin (Figure 1B). Additionally, reduced potencies to four other chemotherapy agents, paclitaxel, Adriamycin, topotecan, and etoposide were observed in A2780-cis cells compared to the sensitive A2780 cells (Figure S1, A–D). Therefore, this A2780-cis cell line is cross-resistant to the commonly used chemotherapy agents.

We then carried out a quantitative drug repurposing combination screen with the addition of 1 μM cisplatin in the A2780-cis cell medium. Cisplatin alone did not significantly reduce the cell viability (due to the drug resistance), but allowed for identification of potential synergistic compounds, which resensitize ovarian cancer cells to cisplatin. The compounds that directly suppress drug-resistant cancer cells can also be found using this method. Thus, this screening approach allows for identification of both single compounds and those that synergize with cisplatin against A2780-cis cell in one compound screening experiment. A total of 6060 compounds consisting of approved drugs and bioactive compounds were screened at five different concentrations for each compound in the presence of 1 μM cisplatin (Figure 1C) that resulted in 383 primary hits (Table S1). Thus, this primary compound screen revealed a group of novel compounds with activities to overcome the drug resistance in A2780-cis cells.

**Identification of Potent Lead Compounds that Suppressed Cisplatin-Resistant Ovarian Cancer Cells**

To further narrow down the active compounds found in this screen, we first performed compound confirmation experiments with the primary hits in the absence of cisplatin. A set of 38 potent compounds inhibited the growth of cisplatin resistant A2780-cis cells (IC₅₀ values <1 μM in the absence of cisplatin) (Table 1). Among these 38 compounds, the anti-cancer activity of five compounds had not been previously reported, whereas the other 33 were anti-cancer compounds, but had not been used in ovarian cancer. A clustering analysis of these compounds based on their clinical indications and known protein targets (Table 1 and Figure 2) revealed that 79% of them were known anticancer agents and the remaining 21% were antibiotics, antifungals, and others (Figure 2A). Most of the targets (68%) were kinase inhibitors including phosphoinositide 3-kinase (PI3K), cyclin-dependent kinase (CDK), and checkpoint kinase 1 (CHK1). The other targets included inhibitors of proteasome components (8%), heat shock proteins (Hsp) (5%), and tubulin depolymerization (5%), while the remaining 14% have other functions or their functions are unclear (Figure 2B).

The five top lead compounds (Figure 3A) may have clinical potential as their in vivo plasma concentrations (Cmax) are higher than the IC₅₀ values, including SR-3306 (IC₅₀ = 0.046 μM) [18], SNX-5422 (0.23 μM) [19], AT-13387 (0.50 μM) [20], GSK-923295 (0.79 μM) [21], PF-05212384 (1.00 μM) [22] (Figure 3B and Table 2). Except SR-3306 (JNK inhibitor), the other four compounds including SNX-5422 (Hsp90 inhibitor), AT-13387 (Hsp90 inhibitor), GSK-923295 (CENP-E inhibitor), and PF-05212384 (PI3K and mTOR dual inhibitor) were tested in early clinical trials. Together, the results revealed potential mechanisms and new drug targets for drug-resistant ovarian cancer, warranting further studies. These five potent compounds with clinical relevance will be useful for studies in animal models and clinical trials.

**Top Five Active Compounds in Combination With Cisplatin Resensitized Cisplatin’s Response in Drug-Resistant Ovarian Cancer Cells**

The primary hits (383 compounds) were also examined for the synergistic effects with cisplatin in the drug-resistant A2780-cis cells in the presence of 0, 6, 12 and 18 μM cisplatin, respectively (a heatmap is shown in Figure 4A). Twelve compounds suppressed the drug-resistant ovarian cancer cells in the combination with cisplatin. Among them, CUDC-101 (EGFR inhibitor), OSU-03012 (PDK1 inhibitor), Oligomycin A (ATP synthase inhibitor), VE-821 (ATM/ ATR inhibitor), and Torin2 (mTOR inhibitor) significantly resensitized cisplatin’s dose–responses in A2780-cis cells determined by the ATP content viability assay (Figure 4), which were also confirmed by the alamarBlue cell viability assay (Figure 5). Because human plasma concentrations of these five compounds are much higher than the in vivo plasma concentrations, these compounds are promising candidates for clinical trials.
higher than the IC50 values obtained in this study, the two-drug combination of cisplatin with these compounds has the potential to be moved into animal models and clinical trials to treat cisplatin-resistant ovarian cancer.

Increases of Phosphorylated-EGFR In the Drug-Resistant A2780 Cells were Reduced by CUDC-101 Treatment

Because the five compounds are either approved drugs or known bioactive compounds, we looked into their potential mechanisms of action. The known properties of these compounds may implicate the pathophysiology of drug resistance and mechanism of cisplatin resensitization in ovarian cancer cells. CUDC-101, a potent inhibitor of EGFR, human epidermal growth factor receptor 2 (HER2), and histone deacetylase (HDAC) [23,24], improved the cisplatin response in A2780-cis cells. A group of 383 hits from the primary screen were selected for confirmation in the same assay, in the presence of vehicle, 6 μM, 12 μM, or 18 μM cisplatin; 11 various concentrations of cisplatin were further combined with the 12 compounds at IC25, IC50, or IC75 for evaluation of combinational effects. Lastly, the above combinational studies were validated for the five candidates in a secondary alamarBlue® viability assay. All values represent the mean ± the standard error of the mean (SEM) (n = 3 replicates).

Figure 1. Platinum drug-resistant ovarian cancer cells and quantitative combination drug screens. (A and B) Concentration-response curves showing the inhibition effect of cisplatin and carboplatin treatment on the viability of both sensitive (A2780) and resistant (A2780-cis) ovarian cancer cells. (C) The primary screens of 6060 compounds from the Library of Pharmacologically Active Compounds (LOPAC), the National Center for Advancing Translational Sciences (NCATS) Chemical Genomics Center Pharmaceutical Collection (NPC), and the Mechanism Interrogation PlatE (MIPE) library were carried out in A2780-cis cells using an ATP content viability assay. Each compound was tested at five concentrations in combination with 1 μM cisplatin. A group of 383 hits from the primary screen were selected for confirmation in the same assay, in the presence of vehicle, 6 μM, 12 μM, or 18 μM cisplatin; 11 various concentrations of cisplatin were further combined with the 12 compounds at IC25, IC50, or IC75 for evaluation of combinational effects. Lastly, the above combinational studies were validated for the five candidates in a secondary alamarBlue® viability assay. All values represent the mean ± the standard error of the mean (SEM) (n = 3 replicates).

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Resensitization of A2780-cis Cells to Cisplatin by Three Other EGFR Inhibitors

To confirm the EGFR inhibitor-specific effect of restoring the A2780-cis cisplatin response, we tested the combination therapy of other EGFR inhibitors. Similar to CUDC-101, three other EGFR inhibitors (WZ4002, varlitinib, and canertinib) resensitized A2780-cis cells to cisplatin (Figure 7, A–C). Specifically, WZ4002 [25], a
Table 1. Compounds with potent activity (IC50 < 1 μM) against A2780-cis ovarian cancer cells

| Compound Name | IC50 (μM) | Function class | Primary activity |
|---------------|-----------|----------------|-----------------|
| Ammonium pyrrolidinedithiocarbamate * | 0.007 | An antioxidant and an inhibitor of NF-kB | Anticancer |
| Dipyridamole * | 0.017 | Blocking proton pump | Antifungal |
| RGB-26147 * | 0.021 | CDK1/2/3/7/9 inhibitor | Anticancer |
| BS-194 * | 0.042 | CDK1/2/5/9 inhibitor | Anticancer |
| SR-3306 * | 0.046 | JNK 1/2/3 inhibitor | Antineurodegeneration |
| CEP-68474 * | 0.096 | CDK1/2 inhibitor | Anticancer |
| CHIR-124 * | 0.105 | Chk1 inhibitor | Anticancer |
| Quinostat hydrochloride * | 0.118 | HDAC1 inhibitor | Anticancer |
| A9 960 * | 0.132 | Jak2/3 inhibitor | Anticancer |
| TCS JNK 3a* | 0.162 | JNK 2/3 inhibitor | Anticancer |
| Ipiinone * | 0.191 | Kinesin-like spindle protein inhibitor | Anticancer |
| SNX-5422 * | 0.234 | Heat shock protein 90 (hsp90) inhibitor | Anticancer |
| Torin2 * | 0.235 | mTORC1 inhibitor | Anticancer |
| Thiram * | 0.296 | Not clear | Antifungal |
| BAY-80-6946 * | 0.332 | PI3K alpha/delta inhibitor | Anticancer |
| NVP-BGT226 * | 0.332 | PI3K inhibitor | Anticancer |
| BMS-3 * | 0.372 | LIMK inhibitor | Anticancer |
| PRI-402 * | 0.418 | PI3K inhibitor | Anticancer |
| GNE-477 * | 0.469 | PI3K inhibitor | Anticancer |
| OAC1 * | 0.469 | Orc1 activator | Enhance reprogramming efficiency |
| AT-13387AU * | 0.505 | Heat shock protein 90 (hsp90) inhibitor | Anticancer |
| PTK-70 | 0.526 | PI3K inhibitor | Anticancer |
| GSX-61364A * | 0.526 | Polo-like kinase-1 (PLK-1) inhibitor | Anticancer |
| Delanoximib * | 0.590 | Proteasome inhibitor | Anticancer |
| MLN-2238 * | 0.662 | Proteasome inhibitor | Anticancer |
| MG-115 * | 0.662 | Proteasome inhibitor | Anticancer |
| Nanchangmycin * | 0.662 | Polyether antibiotic | Antibiotics |
| 2-Fluoroadenosine * | 0.679 | Purine-nucleoside phosphorylase inhibitors | Anticancer |
| AZ-628 * | 0.743 | Raf kinase B/C inhibitor | Anticancer |
| Takeda-6d * | 0.743 | VEGFR-2 (FLK-1/KDR) inhibitors | Anticancer |
| GSX-923295 * | 0.793 | Centromere associated protein (CENP) inhibitors | Anticancer |
| Resitorycin * | 0.833 | RNA polymerase inhibitor | Antibiotics, Anticancer |
| LLL-12 * | 0.855 | STAT-3 inhibitor | Anticancer |
| Proscillaridin * | 0.888 | Steroid | Cardiac glycosides, Anticancer |
| Leucillin hydrochloride * | 0.935 | Tubulin depolymerization inhibitor | Anticancer |
| Parbendazole * | 0.935 | Tubulin depolymerization inhibitor | Anticancer |
| E-7010 * | 0.935 | Tubulin polymerization inhibitor | Anticancer |
| PF-05212384 * | 1.000 | mTOR inhibitor | Anticancer |

Note: IC50 refers to the half-maximum inhibitory concentrations determined from at least 3 independent experiments using A2780-cis ovarian cancer cells.

* indicates compounds have not been previously reported for activity against ovarian cancer, specifically, when last checked in the National Center for Biotechnology Information (NCBI) database in November of 2017.

Discussion

Although chemotherapy is effective for treating ovarian cancer, a majority of patients will eventually relapse and become resistant to platinum-based therapies [28]. Treatment of drug-resistant ovarian cancer is still a challenge. Identification of cisplatin resistance mechanisms helps discover new therapeutics to overcome cisplatin resistance in ovarian cancer. Here, we have developed a quantitative drug combinational screening approach to rapidly identify both single active drugs and two-drug combinations to resensitize the response of drug-resistant ovarian cancer cells. Because approved drugs and bioactive compounds with known mechanisms are used in compound screening, the recognized targets of active compounds can facilitate understanding of drug resistance mechanisms. The 6060 compounds used in this screen include approved drugs, clinical drug candidates, and bioactive compounds [17,29]. While the approved drugs can be novel mutant-selective (L858R)/(T790 M) EGFR inhibitor that does not inhibit HER2, completely resensitized A2780-cis cells to cisplatin. The IC50 value of cisplatin in the drug-resistant cells was decreased by 16-fold in the presence of WZ4002 compared to cisplatin used alone (Figure 7A). Similarly, the synergistic effect of varlitinib and canertinib with cisplatin in the A2780-cis cells was observed (Figure 7B and C). Varlitinib, which is in ongoing Phase III clinical trials [26], is a selective and potent EGFR and HER2 inhibitor. Canertinib, a discontinued clinical candidate [27], is an inhibitor for EGFR and HER2. Together, the results confirmed EGFR inhibitor-mediated cisplatin resensitization in drug-resistant A2780-cis cells by three other EGFR inhibitors.

Knockdown of EGFR Expression Resensitized A2780-cis Cells to Cisplatin

To further confirm the role of EGFR and p-EGFR in A2780-cis cell cisplatin resistance, we carried out a knock-down of EGFR expression using small interfering RNAs (siRNAs). The application of EGFR siRNAs significantly reduced the protein expression of EGFR and its p-Tyr1068 form (Figure 8A–C). Among the three EGFR siRNAs used, the EGFR-3 siRNA reduced EGFR expression to lower levels compared to the other two EGFR siRNAs. Additionally, the HER2 mRNA expression was reduced by all three EGFR siRNAs (Figure 8D). The response of A2780-cis cells to cisplatin was partially recovered after EGFR knockdown (Figure 8E). Like the addition of EGFR inhibitors, the downregulation of EGFR expression and decreased EGFR phosphorylation lessened the resistance of A2780-cis cells to cisplatin. Therefore, the result of the EGFR siRNA knockdown supported EGFR inhibition as the mechanism by which A2780-cis cells were resensitized to cisplatin.
rapidly advanced to clinical trials for new indications, the bioactive compounds may provide opportunities to develop new strategies to overcome cisplatin and other drug resistance. Four of the candidates discussed in this paper—SNX-5422 [19], AT-13387 [20], GSK-923295 [21], and PF-05212384 [22]—have been or currently are being tested in clinical trials for several other cancers. Now, we have found that they could be useful for treating cisplatin-resistant ovarian cancer.

In this study, we added a low clinically relevant concentration of cisplatin (1 μM, does not significantly suppress the drug resistant A2780-cis cells) to our primary compound screen that allowed us to identify two types of compounds that either acted by themselves or in combination with cisplatin against the drug resistance cancer cells. These two types of active compounds can be separated in the hit follow-up studies where the concentration-responses of individual hits are performed in the presence or absence of varying concentrations of cisplatin [30–32]. This quantitative approach not only improves the chance of identifying these two types of hits from one-compound screens, but also significantly reduces false positives caused by the biphasic responses of some compounds. Another quantitative combination screening method involves the use of multiple concentrations of drugs used in standard therapy and compounds identified from the screen [33,34]. One advantage to using this approach is the increased information generated from the screen; information is available with dose–response data in two dimensions for both compounds in the two-drug combination. A

**Figure 2.** Distribution of known drug indications and targets and/or pathways of 38 newly identified potent compounds against cisplatin-resistant ovarian cancer. (A) Number of active compounds in each drug class. If a compound has more than one indication, it is counted once by the following order: anticancer, antibiotic, antifungal, or others. (B) Number of active compounds in each known drug targets/pathways; some compounds have more than one designation.

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**Figure 3.** Five clinically relevant potent anticancer hits are confirmed. Chemical structures (A) and dose–response curves (B) in ATP content viability assays showing the anticancer activities of GSK-923295, SNX-5422, AT-13387 AU, PF-05212384, and SR-3306 against cisplatin-resistant ovarian cancer cell. All values represent the mean ± SEM (n = 3 replicates).
limitation of this matrix-based approach is the enormous amount of resources needed to perform and analyze this type of combinational screen. In our experience, using a single clinically relevant concentration, such as the steady state human plasma drug concentration of a known drug in combination with dose–response curves of individual compounds identified from the primary compound screen permits a rapid discovery of clinically useful two-drug combinations.

Table 2. IC_{50} values against the drug-resistant ovarian cells and their reported concentrations in serum (C_{max})

| Compound      | IC_{50} (μM) | C_{max} (μM) | Reference |
|---------------|-------------|-------------|-----------|
| SR-3306       | 0.046       | 0.34        | [18]      |
| SNX-5422      | 0.23        | 2.42        | [19]      |
| AT-13387      | 0.50        | 9.25        | [20]      |
| GSK-923295    | 0.79        | 13.9        | [21]      |
| PF-05212384   | 1.00        | 16.2        | [22]      |

Figure 4. Combinational anticancer activities of cisplatin and 500 hits. (A) Heatmap showing the change of IC_{50} of hits in the presence of 0, 6, 12, or 18 μM of cisplatin. (B) Magnified heatmap showing the five compounds selected for follow-up studies. (C-G) Chemical structures and dose–response curves in ATP content viability assays showing the improved anticancer activities of cisplatin against drug-resistant ovarian cancer cells in combination with CUDC-101 (C), OSU-03012 (D), Oligomycin A (E), VE-821 (F), and Torin2 (G). All values represent the mean ± SEM (n = 3 replicates).
This compound screen identified and confirmed 38 potent compounds with IC$_{50}$ values less than 1 μM that act by themselves, as well as five two-drug combinations that resensitized ovarian cancer cells to cisplatin. In addition, we also found several less potent compounds (IC$_{50}$ values between 1 and 13 μM, Table S1) that have not been further analyzed, as we only focused on the potent compounds. However, they may still have value for studies of additional drug resistance mechanisms in ovarian cancer, and for identification of additional drug targets that may lead to new therapies.

The top five compounds that exhibited activity as a single compound against the drug resistant A2780-cis cells could be useful for further studies to treat the drug resistant ovarian cancer. SR-3306 is a selective pan-JNK inhibitor with the IC$_{50}$ of 67 nM to JNK1, 283 nM to JNK2, and 159 nM to JNK3, respectively [35,36]. GSK923295 is a potent inhibitor of centromere-associated protein E (CENP-E) that was tested in a Phase-I clinical trial for the treatment of 39 patients with solid tumors [21]. SNX-5422, a pro-drug of SNX-2112 (a selective HSP90 inhibitor), was tested in a clinical Phase-I trial of 56 solid tumor patients. It is currently used in combination with ibrutinib for a clinical trial to treat the chronic lymphocytic leukemia (ClinicalTrials.gov Identifier: NCT02973399) to overcome the drug resistance to ibrutinib (imbruvica), a Bruton’s tyrosine kinase (BTK) inhibitor.

![Figure 5](image1.png)  
**Figure 5.** Confirmation of combinational anticancer activities of cisplatin and five hits in alamarBlue® viability assays. Dose–response curves in viability assays showing the improved anticancer activities of cisplatin against resistant ovarian cancer cell in the combination with CUDC-101 (A), OSU-03012 (B), Oligomycin A (C), VE-821 (D), and Torin2 (E). All values represent the mean ± SEM (n = 3 replicates).

![Figure 6](image2.png)  
**Figure 6.** Inhibitory effects of CUDC-101 on EGFR, p-EGFR, and HER2 in cisplatin-resistant ovarian cancer cells. (A) Western blot of EGFR, p-EGFR, and HER2 expressions in both cisplatin sensitive ovarian cancer cells and resistant ovarian cancer cells. (B) Western blot of EGFR, p-EGFR (Tyr1068), and HER2 expressions after treatment with cisplatin, CUDC-101, or a combination of both in cisplatin-resistant ovarian cancer cells. All experiments are repeated at least three times with a representative blot shown.
tyrosine kinase (BTK) inhibitor. AT-13387 (onalespib) is a selective Hsp90 inhibitor that was tested in a clinical Phase I trial to treat the patients with advanced solid tumors [20]. It is currently being tested in a clinical Phase II trial in combination with paclitaxel for the treatment of patients with advanced triple negative breast cancer (ClinicalTrials.gov Identifier: NCT02474173). PF-05212384 (gedatolisib) is a potent dual inhibitor of PI3K and mTOR that was tested and passed a phase-I clinical trial [22]. Gedatolisib being used in the Phase Ib/II trial as a single agent or in combination with hydroxychloroquine for prevention of recurrent breast cancer (ClinicalTrials.gov Identifier: NCT03400254).

Analysis and characterization of genetic mutations have been widely used to identify of mechanisms of drug resistance during chemotherapy [37,38]. Many mutations in tumor cells like those in protein kinases have been reported to be linked to drug resistance after chemotherapy. In ovarian cancer, an EGFR exon 4 deletion mutant has been found to confer chemoresistance and invasiveness [39]. However, the mechanisms of drug resistance in cancer chemotherapy involve multiple factors and targets other than mutations in one protein. Overexpression or down-regulation of cellular signaling proteins have also been reported in drug-resistant cancer cells [40,41]. Accordingly, constitutive activation of HER2 and HER3 signaling in vitro are correlated with sensitivity to the EGFR inhibitor gefitinib. An alternative method to genetic screens is to use a pharmacological tool to probe the potential mechanisms of action for drug resistance in cancer cells.

The compound screen carried out in this study identified several active compounds against drug-resistant ovarian cancer cells. Known

Figure 7. Combinational effects of cisplatin with other EGFR inhibitors in resistant ovarian cancer cells. (A-F) Dose–response curves showing the inhibition effect of cisplatin in combination with WZ4002, varlitinib, and canertinib on the viability of resistant ovarian cancer cells. All values represent the mean ± SEM (n = 3 replicates).

Figure 8. Improved response to cisplatin in EGFR knock-down resistant ovarian cancer cells. (A) Western blot of EGFR, p-EGFR, and HER2 expressions after treatment with three individual EGFR-siRNA in cisplatin-resistant ovarian cancer cells. (B-D) Quantitation of EGFR (B), p-EGFR (C), and HER2 (D) expression change after treatment with EGFR-siRNA in cisplatin-resistant ovarian cancer cells. (E) The results showed the EGFR-siRNA-3 transfection can decrease the EGFP, p-EGFP and HER2 expression, and the EGFR-siRNA-2 transfection can decrease the p-EGFP expression, and the EGFR-siRNA-1 transfection has no effect. Dose–response curves showing the inhibitory effect of cisplatin on the viability of both sensitive and resistant ovarian cancer cells with/without EGFR-siRNA treatment. All values represent the mean ± SEM (n = 3 replicates).
targets and mechanisms of action of these compounds (facilitated by using approved drugs and bioactive compounds in the libraries) offer good starting points for further investigation of the mechanisms of drug resistance and development of new therapies. For example, CUDC-101, an inhibitor of multiple kinases including HDAC, EGFR, and HER2 [23], was found in our study to restore the cisplatin response in drug-resistant ovarian cancer cells. Following this lead, we performed experiments to confirm EGFR was significantly overexpressed and hyperphosphorylated in cisplatin-resistant A2780-cis ovarian cancer cells; levels of HDAC and HER2 did not change. Indeed, Granados et al. demonstrated that EGFR inhibition by AG1478 and erlotinib during the acquisition of cisplatin resistance in OVCA 433 cells reduced the amount of resistance suggesting EGFR inhibitors may be beneficial to treat platinum resistance in ovarian cancer [42]. Furthermore, knockdown of EGFR in vivo using siRNA in combination with cisplatin treatment significantly reduced ovarian cancer growth [43]. Interestingly, overexpression of EGFR is documented in up to 70% of ovarian cancer patients [44]. However, targeting this pathway by EGFR inhibitors or anti-EGFR antibodies alone showed little efficacy in ovarian cancer patients in clinical trials [45]. One Phase-II clinical trial for erlotinib in combination with cisplatin/paclitaxel found no benefit overall, but a small proportion of patients did show pathological complete response [46]. One argument for these failures is the presence of alternative pathways and signaling architecture with which the cells use to circumvent EGFR inhibition [47]. Another study in vitro using head and neck squamous cell carcinoma and one platinum resistant cervical squamous cell carcinoma line ME-180P found that the drug treatment order impacts the resistance to cisplatin and suggests EGFR inhibitors should not be given prior to cisplatin as this prevents effective degradation of EGFR [48]. Our results have expanded this knowledge with the two-drug combination (an EGFR inhibitor and cisplatin) for treatment of drug-resistant ovarian cancer to overcome the drug resistance caused by overabundance or overactive EGFR.

Although the results of EGFR knockdown with siRNA reduced cisplatin resistance in the drug-resistant cells, it did not fully resensitize cancer cells to cisplatin. This may be caused by an incomplete knockdown of EGFR expression by siRNA in our experiments. Residual EGFR expression after the siRNA knockdown compromised the full efficacy of resensitization that was observed in the experiments with some EGFR inhibitors. We also observed that different EGFR inhibitors exhibited varied efficacy of resensitization to cisplatin in drug-resistant cells. The EGFR inhibitor WZ4002 showed the best effect that completely reversed cisplatin resistance, whereas some other EGFR inhibitors exhibited incomplete activity. This might be caused by the different potencies of these EGFR inhibitors or might involve other unknown kinases; this question needs additional investigation. Importantly, we found not all EGFR inhibitors are equally active in resensitizing cisplatin’s response in the drug-resistant ovarian cancer cells. For example, we found erlotinib and AG1478 were not positive compounds in our compound screening. Supporting this idea, Puvanenthiran et al. found that in combination with paclitaxel, irreversible EGFR inhibitors like canertinib, neratinib and afatinib are more cytotoxic to ovarian cancer cell lines than reversible inhibitors [49]. It is important to note that EGFR inhibitors and EGFR knockdown have differential effects on the cellular signaling architecture. While EGFR inhibitors block the receptor tyrosine kinase activity and the phosphorylation of the cytoplasm facing residues of the C-terminal regions, they do not, in most cases, lead to overall changes in protein expression. On the other hand, knockdown of the kinase using siRNA decreases protein expression outright. RTK serve as scaffolds for many proteins. For example, the SH2 domain of Grb2 and others bind to the phosphorylated Tyr1106 residues of EGFR and ErbB family members at other residues [50].

In conclusion, we demonstrate a quantitative combinational screening method that can rapidly identify both single active compounds and drug combinations against cisplatin-resistant ovarian cancer cells. Because approved drugs and bioactive compounds were used in the screen, the mechanisms of these compounds and synergistic effect of drug combinations can be studied quickly. The clinically relevant single compounds or two-drug combinations can potentially move forward to clinical trials to treat cisplatin-resistant ovarian cancer patients. This approach can be extended to screen active compounds and drug combinations for other drug-resistant cancer cell types, as well as screening of patient-derived primary cancer cells to identify precision treatments.

Materials and Methods

Materials

A2780 human ovarian cancer cisplatin-sensitive cell line (A2780), the A2780 human ovarian cancer cisplatin-resistant cell line (A2780-cis), Opti-MEM®I Reduced Serum Medium (31985070), Lipofectamine® RNAiMAX Transfection Reagent (13778150), VE-821 (SML1415), CUDC-101(EPS003), Torin2 (SML1224), and Oligomycin A (75351), were ordered from Sigma-Aldrich (MO, USA). AG1478 (4,906,837,001, Roche Applied Science, CT, USA), cOmplete, Mini, EDTA-free Protease inhibitor (11836170001), were obtained from PerkinElmer (MA, USA). Alamarblue® cell reagent (12238023), Oligo-Max® RNAiMAX Transfection Reagent (13778150), VE-821 (SML1415), CUDC-101(EPS003), Torin2 (SML1224), and Oligomycin A (75351), were ordered from Sigma-Aldrich (MO, USA). Oligo-Max® RNAiMAX Transfection Reagent (13778150), VE-821 (SML1415), CUDC-101(EPS003), Torin2 (SML1224), and Oligomycin A (75351), were ordered from Sigma-Aldrich (MO, USA). Phosphatase Inhibitor Cocktail Tablets (888001001), NuPAGE™ Bis-Tris Protein Gel (NP0321BOX), M-Per™ Mammalian Protein Extraction Reagent (78505), were purchased from Thermo Fisher Scientific (MA, USA). Phosphatase Inhibitor Cocktail Tablets (888001001), NuPAGE™ Bis-Tris Protein Gel (NP0321BOX), M-Per™ Mammalian Protein Extraction Reagent (78505), were purchased from Thermo Fisher Scientific (MA, USA). Phosphatase Inhibitor Cocktail Tablets (888001001), NuPAGE™ Bis-Tris Protein Gel (NP0321BOX), M-Per™ Mammalian Protein Extraction Reagent (78505), were purchased from Thermo Fisher Scientific (MA, USA). Materials and methods are available at the following link: [Translational Oncology Vol. 11, No. 4, 2018 Combinational drug screen for resistant ovarian cancer Sima et al. 1061](https://example.com)
from Roche Applied Science (CT, USA). The EGF Receptor antibody (2232C), Phospho-EGF Receptor antibody (Tyr1068) (3777C), HER2 antibody (2165S) and β-Actin antibody (4970S) were all purchased from Cell signaling technology (MA, USA). Luminata Forte Western HRP substrate (WBLUF0500) were obtained from MilliporeSigma (MA, USA). EGFR siRNAs (SR301357) were purchased from Origene Technologies Inc. (MD, USA) with following sequences:

- EGFR siRNA-1 sequence – GGAAAUACCUAUGGCAAG GAA T
- EGFR siRNA-2 sequence – AGCUAGAGAGGGAGAAC GGCG
- EGFR siRNA-3 sequence – CGAGGCGAAUACAGCUUUG GUGCC.

**Cell Culture Methods**

Human ovarian cancer cisplatin-sensitive cell line A2780 cells (Sigma-Aldrich, cat. no. 93112519) and cisplatin-resistant A2780-cis cells (Sigma-Aldrich, cat. no. 93112517) were cultured in T-175 tissue culture flasks with 30 ml growth medium in a humidified atmosphere of 5% CO2, at 37°C. Growth medium was made with RPMI 1640 Medium (GIBCO, USA) with 10% fetal bovine serum (FBS). Growth medium was replaced every other day and cells were passaged at 75% confluence.

**Drug Libraries and High-Throughput Screening**

The National Institutes of Health (NIH) Chemical Genomics Center Pharmaceutical Collection (NPC) was constructed in-house through a combination source of traditional chemical suppliers, specialty collections, pharmacies, and custom synthesis. Briefly, the NPC library comprises 2860 small-molecule compounds, 49% of which are drugs approved for human or animal use by the US Food and Drug Administration (FDA), 23% are drugs approved in Canada/UK/EU/Japan, and the remaining 28% are compounds that have entered clinical trials or are research compounds commonly used in biomedical research. The library of mechanism based bioactive compounds was built internally; it also contained some approved antibiotics and allowed to grow overnight to 50% confluence at the time of transfection. For each well to be transfected, 20 pmol siRNA was diluted in 150 μl Opti-MEM® Reduced Serum Medium, and 6.25 μl Lipofectamine™ RNAiMAX Transfection was diluted in 150 μl Opti-MEM® Reduced Serum Medium. The diluted RNAi duplex was mixed with the diluted Lipofectamine™ RNAiMAX gently and incubated for 5 mins at room temperature. The RNAi duplex-Lipofectamine™ RNAiMAX complex was added to each well with cells in a final volume of 2 ml (including 1.7 ml of medium) and a final RNA concentration of 10 nM. Mixing was carried out gently by rocking the plate back and forth. The cells were incubated for 72 hours at 37°C in the incubator.

**siRNA Transfections**

SiRNA knockdown studies were performed as previously described [63]. For transfections, the A2780-cis cells were plated in 6-well plates at a density of 3x10^5 cells per well in 2 ml of growth medium without antibiotics and allowed to grow overnight to 30–50% confluent at the time of transfection. For each well to be transfected, 20 pmol siRNA was diluted in 150 μl Opti-MEM® Reduced Serum Medium, and 6.25 μl Lipofectamine™ RNAiMAX Transfection was diluted in 150 μl Opti-MEM® Reduced Serum Medium. The diluted RNAi duplex was mixed with the diluted Lipofectamine™ RNAiMAX gently and incubated for 5 mins at room temperature. The RNAi duplex-Lipofectamine™ RNAiMAX complex was added to each well with cells in a final volume of 2 ml (including 1.7 ml of medium) and a final RNA concentration of 10 nM. Mixing was carried out gently by rocking the plate back and forth. The cells were incubated for 72 hours at 37°C in the incubator.

**Data Analysis and Statistics**

All data are presented as the mean ± standard error of the mean (SEM) and represent data from three or more independent experiments. The primary screen data was analyzed using customized software developed internally [64]. IC_{50} values were calculated using the Prism 5 software (GraphPad Software, CA, USA). The two-tailed unpaired Student’s test was used for multiple comparisons. A P-value less than 0.05 was considered significantly different.

**Clustering of Compounds by Activity Outcomes**

Compounds were clustered hierarchically using TIBCO Spotfire 6.0.0 (Spotfire Inc., Cambridge, MA) based on their activity outcomes from the primary or follow up screen across different testing conditions. Clustering was done based on a compound’s potency. In the heatmap, potencies were represented in the following categories: ≤0.1 μM, 0.1 to 1 μM, 1 to 10 μM, and 10 to 20 μM, with a darker color indicating compounds that are more potent and efficacious; lighter colors indicating less potent and efficacious compounds. If a compound did not show any activity in an assay, it was highlighted as gray in the heatmap.

**Data availability Statement**

Data will be made available upon request.
Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.06.002.

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Author Contributions
N.S., W.S., and W.H. carried out the experiments. W.S. and W.Z. wrote the manuscript. K.G. and W.Z. revised, edited, and prepared the manuscript for resubmission. M.S. and W.S. analyzed the data. W.S., W.Z., X.X., and X.C. conceived the original idea for the research.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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