Quantitative High-Throughput Screening Using an Organotypic Model Identifies Compounds that Inhibit Ovarian Cancer Metastasis

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ABSTRACT

The tumor microenvironment (TME) is a key determinant of metastatic efficiency. We performed a quantitative high-throughput screen (qHTS) of diverse medicinal chemistry tractable scaffolds (44,420 compounds) and pharmacologically active small molecules (386 compounds) using a layered organotypic, robust assay representing the ovarian cancer metastatic TME. This 3D model contains primary human mesothelial cells, fibroblasts, and extracellular matrix, to which fluorescently labeled ovarian cancer cells are added. Initially, 100 compounds inhibiting ovarian cancer adhesion/invasion to the 3D model in a dose-dependent manner were identified. Of those, eight compounds were confirmed active in five high-grade serous ovarian cancer cell lines and were further validated in secondary in vitro and in vivo biological assays. Two tyrosine kinase inhibitors, PP-121 and milciclib, and a previously unreported compound, NCGC00117362, were selected because they had potency at 1 μmol/L in vitro. Specifically, NCGC00117362 and PP-121 inhibited ovarian cancer adhesion, invasion, and proliferation, whereas milciclib inhibited ovarian cancer invasion and proliferation. Using in situ kinase profiling and immunoblotting, we found that milciclib targeted Cdk2 and Cdk6, and PP-121 targeted mTOR. In vivo, all three compounds prevented ovarian cancer adhesion/invasion and metastasis, prolonged survival, and reduced omental tumor growth in an intervention study. To evaluate the clinical potential of NCGC00117362, structure–activity relationship studies were performed. Four close analogues of NCGC00117362 efficiently inhibited cancer aggressiveness in vitro and metastasis in vivo. Collectively, these data show that a complex 3D culture of the TME is effective in qHTS. The three compounds identified have promise as therapeutics for prevention and treatment of ovarian cancer metastasis.

Introduction

Approximately 70% of patients with ovarian cancer present with metastatic disease, for which we currently have no available targeted treatments. Indeed, ovarian cancer is the leading gynecologic cancer, with an estimated 17,000 newly diagnosed cases in the United States in 2018 (1). The overall survival rate of patients with advanced high-grade serous ovarian cancer is extremely low despite aggressive treatment with surgery and chemotherapy (2). This poor prognosis is due to widespread metastasis at the time of diagnosis and therapeutic resistance. Therefore, new therapies targeting ovarian cancer metastasis are essential if we are to significantly improve the prognosis for patients with ovarian cancer.

The cellular and extracellular components of the tumor microenvironment (TME) play a critical role in cancer metastasis and drug resistance (3,4). Metastasis of high-grade serous ovarian cancer mainly involves the detachment of ovarian cancer cells from the primary tumor and their subsequent transcoelomic dissemination in the peritoneal fluid to mesothelium-lined metastatic sites within the confines of the abdominal and pleural cavities. The ovarian cancer tumor cells and the cells in the surrounding peritoneal tumor microenvironment interact closely and play a critical role in the progression and metastasis of ovarian cancer (reviewed in refs. 5–7). For the past several years, we and others showed that adipocytes (8), fibroblasts (9), macrophages (10), and mesothelial cells (11–15) change their phenotype to cancer-associated stromal cells that promote ovarian cancer cell growth by bidirectional communication.

The first organotypic model that recapitulated the mesothelium surface histology of the omentum and peritoneum was based on a detailed analysis of the omentum and peritoneum of patients without ovarian cancer (16). Recently, this model was further developed to perform high-throughput drug screening (HTS) in 384- and 1,536-well format to screen 2,000 compounds (17,18). Specifically, primary human omental mesothelial cells and fibroblasts are mixed with extracellular matrix (ECM) to reconstruct, in vitro, the superficial tissue layer of the mesothelium, the principal site of ovarian cancer metastasis (19–21).

The goal of this study was to identify inhibitors of ovarian cancer metastasis by performing a fully robotic qHTS cell-based assay using five high-grade serous ovarian cancer cell lines, primary human stromal cells, and ECMS. Over 44,000 new, structurally diverse small-molecule compounds and clinical drugs were screened using the complex 3D qHTS assay. Through a series of confirmatory and secondary in vitro and in vivo assays, three compounds that inhibit ovarian cancer metastasis in vivo were identified.

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Materials and Methods

Reagents

Two collections of compounds were screened at the NCATS were screened: A collection of 44,420 diverse small-molecule drugs; and the Mechanism Interrogation Plate 4.0 collection of 386 pharmacologically active small molecules (22). The active compounds were repurchased for repeat and secondary assays. Milciclib (catalog #PHA-848125), MK-0752 (#HY-10974), and PP-121 (#HY-10372) were purchased from MedChemExpress. Ouabain (#1076) and AMG-51 (#SYN-111) were purchased from Thermo Fisher Scientific. Vardenafil (#Y000167) and oridonin (#09639) were purchased from Sigma-Aldrich. Peruvoside (#P227570) was purchased from Toronto Research Chemicals. NCCG00117362 (#D233-0871), NCCG00117361 (#C668-0165), NCCG00117328 (#D233-0834), NCCG00117305 (#D244-0327), NCCG00117477 (#D234-0252), NCCG00117166 (#D233-0497), NCCG00115018 (#D053-0260), NCCG00117330 (#D233-0835), and NCCG00117364 (#D233-0885) were purchased from ChemDiv. Collagen type I (rat-tail), and fibronectin (human) was purchased from BD Biosciences. Rabbit anti-phospho m-TOR against Ser2448 (#2971), rabbit anti-mTOR (#2972), rabbit anti-phospho Rb against Ser112 (#9309), mouse anti-Cdk6 (#3136), lysis buffer (#803), and 3X Blue Loading Buffer (#56036) were purchased from Cell Signaling Technology. Mouse anti-Cdk1/Cdk2 (#sc-53219) was purchased from Santa Cruz Biotechnology Inc.

Cell lines

The Ovcar4 cells were purchased from the DCTD Tumor/Cell Line Repository (#0507673). The COV318 cells were obtained from Gottfried Konczeny at UCLA (Los Angeles, CA). Dr. Gordon B. Mills (originally at M.D. Anderson Cancer Center, Houston, TX) provided the SKOV3ip1 and HeyA8 cells. The CaOv3 cells were purchased from the ATCC (#HTB-75). The Ovcar5 cells were obtained from UCSF (23). The Tyk-nu cells were obtained from UCLA (24). The Kuramochi cells were purchased from the JCRB Cell Bank (#JCRB0098). The Snu-119 cells were purchased from the Korean Cell Line Bank (#00119). All of these ovarian cancer cell lines were cultured in DMEM with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 10% FBS, 1% insulin-transferrin-selenium, 1% L-glutamine and 1% penicillin-streptomycin (absent in CETSA assays). The 59M cells were obtained from the European Collection of Cell Cultures and cultured in DMEM with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 10 µg/mL insulin, and 10% FBS. The GFP luciferase-labeled ovarian cancer cells were constructed utilizing a lentivirus vector expressing coppep GFP (#CD511B-1) and the Lentivirus Packaging Kit (#LV500A-1) from System Biosciences (9). The Id1/Id2/Id3/Id4 were provided by Ian McNeish (25) and are cultured in DMEM with 4% FBS, 1× insulin-transferrin-selenium, 1% L-glutamine and 1% penicillin-streptomycin. All cell lines were passaged 3 to 8 times after thawing before used in described experiments. The cell lines were banked in liquid nitrogen and one vial of each passage were confirmed Mycoplasma negative using the STAT-Myco Kit and validated using short tandem repeat DNA fingerprinting with the AmpF/STR Identifier Kit and compared with known fingerprints by IDEXX BioAnalytics Laboratories.

Primary HTS assay

Specimens of fresh human omentum were obtained from patients undergoing surgery for benign conditions who gave written informed consent before surgery. The protocol was approved by the University of Chicago Institutional Review Board. Primary human mesothelial cells and fibroblasts were isolated from normal human omentum; purity was verified by vimentin, calretinin, and cytokeratin IHC and used within two passages of isolation (12, 16).

For the 1,536-well format, 40 primary human omental fibroblasts and 400 mesothelial cells were seeded with 0.02 µg fibronectin and 0.02 µg collagen type I in 4 µL of growth media (2.3 mm², Supplementary Fig. S1A). After a 48-hour incubation at 37°C, 1,200 SKO-V3ip1-GFP were seeded in 3 µL of serum-free media (growth media minus FBS) on top of primary human omental cells. The compounds were screened in four doses (0.36–46 µmol/L) and the plates contained the positive control (Tomatine) in eight doses (0.035–75 µmol/L) and DMSO (equal volume controls). The compounds or controls were added to each well immediately after addition of the cancer cells. The plates were incubated at room temperature for 2 hours and then at 37°C for 16 hours. Following incubation, the media were aspirated and each well washed with PBS (5 µL) and then fixed with 4% paraformaldehyde (PFA; 5 µL). After 15 minutes, the PFA solution was aspirated, PBS (5 µL) was added, and the number of GFP-labeled cells analyzed using a fluorescent cytometer (TTP LabTech Acumen x3).

Confirmatory and counter assays

The 3D HTS assay was plated in a 384-well format. Primary human omental fibroblasts (400) and mesothelial cells (4,000) were seeded with collagen type I in 40 µL of growth media (0.06 cm²). After 48-hour incubation at 37°C/5% CO2/95% relative humidity, SKOV3ip1-GFP, HeyA8-GFP, Kuramochi-GFP, COV318-GFP, SNU119-GFP, Tyk-nu-GFP, or Ovcar4-GFP ovarian cancer cells (12,000) were seeded in 40 µL of serum-free media (growth media minus FBS) on top of primary human omental cells (final volume of 80 µL total). The library compounds dissolved in DMSO at 46 µmol/L final concentration, positive control (Tomatine) at 10 µmol/L final concentration, or DMSO (0.5% final concentration) were added to each well immediately after the addition of cancer cells. The plates were incubated at room temperature for 2 hours before being placed in an incubator at 37°C. After a 16-hour incubation, each well was washed with PBS (40 µL) followed by fixation with 4% paraformaldehyde. The number of fluorescent cells in the assay was analyzed using a fluorescent cytometer (TTP LabTech Acumen x3).

For the confirmatory assay, the dose response of the compounds was tested using 12 concentrations (10 nmol/L–100 µmol/L). The compounds dissolved in DMSO or DMSO (equal volume control) were added to each well immediately after the addition of cancer cells. The plates were incubated, treated, and fixed as described above and the number of fluorescent cells analyzed using the fluorescent cytometer.

Counter assay

Ovarian cancer cells (4,000 SKOV3ip1-GFP or 2,000 HeyA8-GFP) were seeded in 40 µL of growth media and incubated for 24 hours. Compounds dissolved in DMSO at 12 concentrations (10 nmol/L–100 µmol/L) or DMSO (equal volume control) were added. The plates were incubated at room temperature for 2 hours before being placed in an incubator at 37°C. After a 16-hour incubation at 37°C, the CellTiter-Glo cell viability assay (Promega) was performed and analyzed using a luminescent plate reader (BioTek Synergy NEO2).

The compound’s AUC for activity outcomes from the confirmatory and counter screens was calculated on the basis of the data analysis and dose-response curve fittings. The compounds were clustered hierarchically using TIBCO Spotfire 6.0.0 (Spotfire Inc.).

Secondary biological in vitro assays

The secondary biological assays were miniaturized for high-throughput analysis. The 3D culture was assembled on black-walled
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384-well plates for adhesion and growth assays using the iPiPette from Apricot Designs. For invasion assays, the 3D culture was assembled on precoated (7 μg of collagen type I) 96-well transwell inserts (BD Biosciences; refs. 12, 16). The compounds were repurchased as described above. Compounds dissolved in DMSO were tested at 1, 2, 5, and 10 μmol/L concentrations, and DMSO (equal volumes) was the control.

**Adhesion**

A total of 8,000 fluorescently labeled Tyk-nu-GFP, Ovcar5-GFP, or Kuramochi-GFP ovarian cancer cells were mixed with the compounds and seeded in 40 μL of serum-free media on top of the 3D culture (0.33 cm², n = 5–15). After 1-hour incubation at 37°C, the wells were washed with PBS, fixed with PFA, and cell number computed using a Spectramax i3 MiniMax 300 imaging cytometer (Molecular Devices).

**Invasion**

A total of 8,000 Tyk-nu-GFP, Ovcar5-GFP, or Kuramochi-GFP cells were seeded in 40 μL of serum-free media in the upper chamber of a 96-well transwell plate (0.134 cm², n = 5–10) precoated with the 3D culture (12). The compounds were added to the cancer cells in the upper chamber, growth media (100 μL) were placed in the bottom chamber, and the plates were incubated at 37°C for 24 to 48 hours. All cells were removed from the top chamber and the invaded ovarian cancer cells were quantified using the SpectraMax i3 MiniMax 300 imaging cytometer.

**Proliferation**

A total of 2,000 Tyk-nu-GFP, 1,000 Ovcar5-GFP, or 4,000 Kuramochi-GFP cells were seeded in 40 μL of serum-free media on top of the 3D culture (0.33 cm², n = 5–15). The compounds were added after 30 minutes, plates were incubated for 72 hours at 37°C, and total number of cells was counted using the SpectraMax i3 MiniMax 300 imaging cytometer.

**Animal experiments**

Female C57BL/6NCrI (C57BL/6; #027) mice and female HSD: Athymic Nude-Foxn1nu (athymic nude; #069nu/070nu/+ ) mice at age 5 to 6 weeks and approximately 20 g were purchased from Charles River Laboratories and Envigo, respectively. All procedures involving animal care were approved by the Committee on Animal Care at the University of Chicago (Chicago, IL).

**In vivo adhesion/invasion assay**

Mice were randomized into groups (n = 5) and injected intraperitoneally with 4 × 10⁶ ID8p53−/− luciferase/GFP cells mixed with compounds (5 μmol/L) or DMSO (equal volume) on day 1. Mice were treated twice after inoculation (48 and 96 hours) with intraperitoneal injection of compounds (1 mg/kg/day) in PBS (200 μL). The mice were sacrificed at the first signs of distress (17).

**In vivo intervention assay**

Mice were injected intraperitoneally with 4 × 10⁶ ID8p53−/− luciferase/GFP cells. Twenty-one days after cancer cell injection, the mice were randomized (n = 5) into groups and treated with intraperitoneal injection of compounds (5 mg/kg/day) or DMSO (equal volume) in PBS (200 μL) daily for 10 days. The mice were sacrificed 1 day after last treatment. The omental tumors were weighed. The concentration of milciclib tested is lower than the doses reported in clinical trials.

**Kinase selectivity assays**

Tyk-nu cells were grown in Corning hyperflasks, washed with PBS, and then treated with milciclib or PP-121 for 16 hours (100, 1, 0.1, and 0.1 μmol/L). The cells were washed with PBS, trypsinized, neutralized, and pelleted. The pellets were snap-frozen in liquid nitrogen and sent to ActiveX Biosciences Inc. to perform in situ kinase profiling (KiNativ; ref. 27).

**ImmunobLOTS**

RIPA buffer was used to lyse cells for cellular p-mTOR, mTOR, pRb, and Rb analysis. Cell Signaling Lysis Buffer was used to lyse cells for pRb and Rb detection. Equal amounts of protein were added to each well of an SDS-PAGE gel and resolved. Proteins were transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk tris-buffered saline with 0.1% tween. The membranes were incubated in primary antibodies 1:1,000 dilution in 5% BSA tris-buffered saline overnight at 4°C. The next day, the blot was incubated with horseradish peroxidase–conjugated IgG secondary antibody at room temperature for one hour at a 1:5,000 dilution, and visualized using chemiluminescence reagents.

**Cellular thermal shift assay**

Tyk-nu cells (3 × 10⁵) were suspended in 100 μL of PBS containing complete Mini EDTA-free protease inhibitor cocktail. To evaluate Cdk2 or Cdk6 aggregation, temperature cell samples in thin-wall PCR stripe were incubated for 3 minutes between 45°C and 65°C using Bio-Rad T100 Thermal Cycler. The negative control sample was incubated at room temperature. Cdk2 or Cdk6 thermal stability upon inhibitor binding was evaluated by incubation of the cell suspensions containing DMSO solutions of inhibitor in 100 μmol/L to 1 nmol/L concentrations and inhibitor solvent control sample at 52°C. Afterwards, samples were centrifuged at 3,000 × g for 40 minutes at 4°C. Supernatants containing nonaggregated fraction of protein were separated by SDS-PAGE (28). Protein was visualized by immunoblotting using mouse anti-Cdk1/Cdk2 and mouse anti-Cdk6 diluted 1:1,000. Intensity of the bands on the membranes was quantified in ImageJ software. The charts of the relative amount of Cdk2 or Cdk6 (normalized to RT control) stabilized in the presence of milciclib or PP-121 in range of concentrations were prepared in GraphPad Prism version 7.04.

**Statistical analysis**

Confirmatory (n = 8), adhesion (n = 5–15), invasion (n = 5–8), and growth (n = 8–15) assays were conducted in at least three independent experiments. The mean and SD or SEM are reported. All statistical analyses were performed using GraphPad Prism (GraphPad). For experiments comparing two groups, data were analyzed using a two-tailed Mann–Whitney U test to account for nonnormal
distribution of the data. For experiments with more than two groups, one-way ANOVA followed by Dunnett multiple comparisons test (DMSO vs. each of the other groups) was used. Differences were considered significant if $P < 0.05$. Kaplan–Meier survival estimates were calculated to assess group differences in the survival study. For the survival assay, a log-rank test was performed to compare the four treatment groups and individual comparisons of DMSO versus treatment were reported (Bonferroni-adjusted).

Results

Use of a 3D organotypic assay for qHTS

Our screening approach to evaluate the effect of small-molecule compounds on the early steps of ovarian cancer metastasis is shown in Fig. 1A. A 3D organotypic assay was implemented onto a fully automated robotic platform and 44,806 compounds screened. In this assay, primary human fibroblasts and mesothelial cells were embedded...
in fibronectin and collagen and allowed to grow for 48 hours in 1,536-well plates. The GFP-labeled cancer cells were then added, followed by compound treatment, and a short incubation at room temperature of 2 hours (Supplementary Fig. S1A and B, Materials and Methods). After an additional 16-hour incubation at 37°C, the adhered/invaded cancer cells were quantified using a fluorescent cytometer, after the nonadhered cells were washed. Each compound was tested at 4 doses, 0.37, 1.8, 9.2, and 46 μmol/L (Fig. 1B and C).

The expansion of the HTS platform for the screening of 44,806 compounds did not compromise the quality of the HTS assay, which was still robust and reproducible with signal-to-background ratios of 22- to 63-fold and Z’-factor values of 0.4 to 0.84 (Supplementary Fig. S1C).

From the primary screen, we identified 378 compounds that inhibited the adhesion/invasion of SKOV3ip1 cells in a dose-dependent manner. In a confirmatory assay, the compounds were retested in the 3D adhesion/invasion HTS assay using a 12-point response in SKOV3ip1 and HeyA8 cells (Supplementary Fig. S2). A counter screen was performed to identify and eliminate compounds that affected ovarian cancer cell viability at a similar doses and time of the assay (Supplementary Fig. S1C).

Consistent with the in vitro studies, all eight compounds inhibited adhesion/invasion to the omentum in vivo (Fig. 4A; Supplementary Fig. S4C).

Efficacy of the compounds in in vivo metastasis assays
The effect of early treatment (prevention study) was tested using three in vivo assays. First, we performed a short-term adhesion/invasion assay in the mouse peritoneal cavity (8, 11, 17), using the same 16-hour time point used in the in vitro studies. Luciferase-labeled ID8p53<sup>−/−</sup> ovarian cancer cells were mixed with each of the eight most efficient compounds (NGCG00117362, PP-121, milciclib, AMG-51, MK-0752, NCGCG00161703, oridonin, and vardenafil), injected intraperitoneally into mice, and cancer cell adhesion/invasion measured. Consistent with the in vitro studies, all eight compounds inhibited adhesion/invasion to the omentum in vivo (Fig. 4A; Supplementary Fig. S4C).
Activity of the two kinase inhibitors in ovarian cancer cells

Because PP-121 and milciclib are both kinase inhibitors, we investigated their kinase selectivity profile. Using in situ kinase profiling of 156 kinases (Fig. 5A), PP-121 in Tyk- nu cells inhibited mTOR and WNK1-3. Using immunoblotting, we confirmed that PP-121 inhibits phosphorylation of mTOR in all three ovarian cancer cell lines (Ovcar5, Kuramochi, and Tyk- nu; Fig 5B). Because multiple mTOR inhibitors are currently being used clinically, we wanted to determine whether other mTOR inhibitors inhibit metastasis. However, early intraperitoneal treatment with everolimus or temsirolimus, two mTOR inhibitors, had no effect on ovarian cancer adhesion/invasion to the omentum in vivo (Supplementary Fig. S5).

Using the same in situ kinase profiling described above, we found that milciclib inhibited a plethora of kinases (Fig. 5C), including cyclin-dependent kinases (CDK)2/4/6. Using cellular thermal shift assay (CETSA) in Tyk- nu cells, we determined that milciclib interacts directly with Cdk1/Cdk2 and Cdk6 (Fig. 5D and E). The downstream effector of CDK is retinoblastoma (Rb) protein, which upon phosphorylation, allows the cell cycle to proceed through G1 (32). In three ovarian cancer cell lines (Ovcar5, Tyk- nu, and Kuramochi; Fig 5F), milciclib inhibited phosphorylation of Rb, which indicates that one of its mechanisms of action is cell-cycle arrest. This inhibition was independent of cyclin E1 levels, because milciclib similarly inhibited the invasion and proliferation of ovarian cancer cells expressing low or high levels of cyclin E1 inhibitor (Supplementary Fig. S6).

Next, we investigated whether milciclib and NCGC00117362 could regulate the phosphorylation of mTOR, or whether PP-121 and NCGC00117362 could regulate the phosphorylation of Rb. Milciclib and NCGC00117362 did not regulate the phosphorylation of mTOR, while PP-121 and NCGC00117362 did not control the phosphorylation of Rb (Supplementary Fig. S7).

Structure–activity relationship of NCGC00117362

The third lead compound, NCGC00117362, has not been previously reported to affect tumor growth or metastasis. Gene expression profiling of ovarian cancer cells after 24 hours of treatment with NCGC00117362 revealed significant changes in the expression of genes associated with NF-kB survival, MAPK signaling, and GPCR ligand binding (Supplementary Table S1). Consistent with the pathway analysis, NCGC00117362, but not PP-121 or milciclib downregulated the expression of Rac2, IL1β, and MMP-14 (Supplementary Fig. S8).

To evaluate the relationship between the chemical structure of this compound and its biological activity, structure–activity relationship (SAR) studies were performed. Four close SAR analogues of the NCGC00117362 compound (Fig. 6A) and three inert control compounds (Fig. 6B) from the same chemical series were identified, purchased, and tested using the screening paradigm described in Fig. 1A. The four SAR analogues significantly inhibited mice (median survival: 30 days). There was no significant difference in survival comparing the three treatment groups.

Finally, the three compounds were tested in an intervention treatment assay in which mice were injected intraperitoneally with ID8p53−/− cells, and 21 days after cancer cell injection, the mice were treated intraperitoneally with compounds (5 mg/kg/day) for 10 consecutive days. The treatment of mice with NCGC00117362, PP-121, and milciclib decreased omental tumor weight significantly (Fig. 4D). NCGC00117362 treatment inhibited omental tumor growth significantly more than PP-121 (P = 0.007) or milciclib (P = 0.002) treatment.

Activity of hit compounds in secondary in vitro biological assays. Secondary in vitro screens (adhesion, invasion, and proliferation) were performed. The effect of compounds at 4 doses was tested in three ovarian cancer cell lines. Ovarian cancer cell adhesion (2 hours; A) and proliferation (96 hours; C) were tested in 96-well plates on the 3D organotypic culture. Ovarian cancer cell invasion (24–48 hours; B) was tested using a 96-well Boyden chamber lined with collagen type I as ECM and the 3D organotypic culture. Mean ± SD. **P < 0.01; ***P < 0.001; ****P < 0.0001; n = 5–8.

Fig. S4D). Because three compounds (NCGC11007362, PP-121, and milciclib) significantly inhibited at least two key in vitro ovarian cancer functions in early metastasis at a low dose (1 μmol/L) and inhibited in vivo ovarian cancer cell adhesion/invasion at 5 μmol/L, we evaluated the efficacy of these compounds further in vivo.

We next tested these three compounds in a metastasis prevention assay using the ID8p53−/− ovarian cancer cells syngeneic model (30, 31). The ovarian cancer cells were mixed with each of the three compounds (NCGC00117362, PP-121, or milciclib) and injected intraperitoneally. After 2 more treatments (day 2, 4), which were followed by 41 days of observation, NCGC00117362, PP-121, and milciclib were found to reduce tumor number by at least 70% and tumor weight by 64% (Fig. 4B). Next, the three compounds were tested in a survival assay (12, 17) in which mice were injected intraperitoneally with ID8p53−/− cells using the same treatment schedule as described for the previous in vivo assay. The treatment of mice with NCGC00117362, PP-121, and milciclib increased overall survival significantly (median survival: 47, 53, and 50 days, respectively; Fig. 4C) when compared with vehicle-treated control
adhesion/invasion, while the three inert control compounds had no effect on the adhesion/invasion of five serous ovarian cancer cell lines to the 3D HTS assay (Supplementary Fig. S9). Interestingly, NCGC00117362 and one other compound, NCGC0017328, inhibited ovarian cancer adhesion (Fig. 6C), while all SAR analogues inhibited ovarian cancer cell invasion (Fig. 6D). Only NCGC00117362 inhibited ovarian cancer proliferation (Fig. 6E). The inert control compounds had no effect on ovarian cancer adhesion, invasion, or proliferation (Fig. 6C–E). Consistent with the in vitro studies, only the four SAR analogues inhibited ovarian cancer adhesion/invasion to the omentum in vivo (Fig. 6F; Supplementary Fig. S5). Furthermore, intraperitoneal treatment of mice with the SAR analogues prior to ovarian cancer cell implantation on the intra-abdominal surface reduced tumor weight and tumor number by at least 30% when compared with control mice (Fig. 6G). Conversely, inert control compounds had no effect on omental tumor growth when treatment was given 21 days after ovarian cancer cell injection in a syngeneic mouse model (Fig. 4D).

Discussion

A previously developed complex organotypic assay that reconstructs important attributes of the ovarian cancer metastatic microenvironment (17, 18) was used to screen 44,802 compounds in qHTS. The compounds screened included new, structurally diverse compounds and existing approved or investigational drugs that could potentially be repurposed for ovarian cancer therapy. This 1,536-well primary assay, simulating the setting of ovarian cancer metastasis and the systematic follow-up approach after the primary screen (Fig. 1A), proved to be successful strategies that were able to identify inhibitors of ovarian cancer metastasis in vivo. The activity of compounds, which blocked at least two ovarian cancer functions (adhesion, invasion, and/or proliferation) in multiple ovarian cancer cell lines in vitro, was confirmed in three in vivo assays using xenograft and syngeneic mouse models with prevention and intervention treatment regimens.

Three compounds with both in vitro and in vivo activity were identified using our screening strategy, the two kinase inhibitors, PP-121 and milciclib, and a new compound, NCGC00117362. Using an unbiased kinase screening platform, we found that PP-121 specifically bound to mTOR and inhibited mTOR phosphorylation at low doses. PP-121 has been reported as a strong multi-targeted kinase inhibitor, with activity against PDGFR, Hck, mTOR, VEGFR2, Src, and Abl. It is also known to block protumorigenic signaling pathways, including Akt-mTOR and NF-κB (33). In glioblastoma cell lines, it has been shown that PP-121 blocks proliferation (33), and in thyroid cancer cell lines, it inhibits thyroid cancer cell migration, invasion, cell viability, and tumor growth (34). Therefore, PP-121 is a dual inhibitor of tyrosine and phosphoinositide kinases that targets major protumorigenic signaling pathways.

Milciclib bound cyclin-dependent kinases at low doses in ovarian cancer cells. Specifically, milciclib interacted with CDK6 and PCTAIRE1 (CDK16). Moreover, milciclib inhibited Rb phosphorylation, which is downstream of CDK activity as previously reported in...
Figure 5.
Kinase selectivity profile of PP-121 and milciclib in ovarian cancer cells. A, Tyk-nu cells were treated with increasing concentrations of PP-121 for 16 hours, and in situ kinase profiling was performed. Dendrogram representation of kinase inhibitor activity. Potency depicted by length of the bar for each kinase, and color code for concentration active. B, Immunoblot analysis of phosphorylated and total mTOR after PP121 (5 μmol/L) or DMSO control treatment for 24 hours in Kuramochi, Ovcar5, and Tyk-nu cells. C, Tyk-nu cells were treated with increasing concentrations of milciclib for 16 hours, and in situ kinase profiling was performed. Dendrogram representation of kinase inhibitor activity. Potency depicted by length of the bar for each kinase, and color code for concentration active. D and E, Cellular thermal shift assays of Cdk1/Cdk2 (D) and Cdk6 (E) with increasing concentrations of milciclib (1 nmol/L–100 μmol/L). F, Immunoblot analysis of phosphorylated and total retinoblastoma (Rb) after milciclib (1 μmol/L) or DMSO control treatment for 24 hours in Kuramochi, Ovcar5, and Tyk-nu cells. RT, room temperature.
melanoma (35), glioma (36), and the A2680 ovarian cancer (37) cell lines. The effect of milciclib on cancer cells is not limited to ovarian cancer; it has also shown efficacy in non–small cell lung, melanoma, colon, pancreatic, and prostate cancer subcutaneous tumor growth in vivo (37) and is currently in phase I and II clinical trials (NCT03109886, NCT01301391, NCT01011439) in patients with unresectable/metastatic hepatocellular carcinoma, thymic carcinoma, and thymoma. These reports, and the data presented here, support the further clinical development of milciclib toward clinical testing in epithelial ovarian cancer.

The third compound, NCGC00117362, is in a group of structurally similar small molecules, which reliably inhibited in vitro and in vivo ovarian cancer activity in SAR studies. NCGC00117362 downregulated multiple signaling pathways, including NF-κB and MAPK,
important signaling pathways in high-grade serous ovarian cancer (38, 39). The efficiency of NCGC00117362 and its four SAR analogues in 

\textit{in vitro} and \textit{in vivo} studies supports our view that the structure of this compound may provide a promising lead for the further development of a candidate that is clinically effective against metastatic disease.

That the NCGC00117362 compound was identified using our systematic 3D approach, and the fact that all analogues also proved efficient against the mechanisms tested with the HTS assay and subsequent \textit{in vitro} and \textit{in vivo} biological studies, shows that our approach can very effectively identify drug candidates for further clinical development. In this study, we proved that a 3D model could be used with high reproducibility in a HTS setting with tens of thousands of compounds. Of note, we established the assay at the University of Chicago and transferred the technology to NCATS, showing that the method may be adapted for various HTS settings in different laboratories. Although the in vivo microenvironment is not fully recreated in this model (it lacks endothelial and immune cells), the model successfully incorporates primary human cells in a dependable, consistent, and reproducible 1,536-well assay format. The modular concept of the 3D model can be used to screen for inhibitors of other cancers by customizing it to simulate the microenvironments specific to those cancers. For example, the effect of different drugs on the specific metastatic sites of castrate-resistant prostate cancer could be investigated by recreating the specific TME of lymph nodes, bone, or liver (40).

In the future, the 3D model could be further developed and individualized by testing drugs using syngeneic primary patient-derived clini- derived clini- and host cells at the time of the initial surgery and diagnosis, establishing a truly individualized therapy. In summary, our complex physiologically relevant model was successfully used in qHTS to identify potential therapeutics for the prevention of ovarian cancer metastasis. If these agents reach clinical use, they could be beneficial for patients after optimal tumor reduction to reduce the reseeding of cancer cells in the abdominal cavity.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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