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Small molecule inhibition of the CBF β /RUNX interaction decreases ovarian cancer growth and migration through alterations in genes related to epithelial-to-mesenchymal transition

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Abstract

Objective—Ovarian cancer survival and treatment have improved minimally in the past 20 years. Novel treatment strategies are needed to combat this disease. This study investigates the effects of chemical inhibition of the CBF β /RUNX protein-protein interaction on ovarian cancer cell lines.

Methods—Ovarian cancer cell lines were treated with CBF β /RUNX inhibitors, and the effects on proliferation, DNA replication, wound healing, and anchorage-independent growth were measured. RNA-Seq was performed on compound-treated cells to identify differentially expressed genes. Genes altered by compound treatment were targeted with siRNAs, and effects on DNA replication and wound healing were measured.

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Author Contributions

A.L.C., Y.G., and A.S. performed experiments; A.L.C. and A.B. analyzed data; A.I., D.C.L., C.N.L., and R.A.R. provided reagents; A.L.C., C.N.L., D.W., and J.H.B. designed experiments; and A.L.C., D.W., and J.H.B. wrote and edited the manuscript.

Conflict of Interest

A.I. and J.H.B. are co-inventors on a patent filing (US20170233375 A1) that includes compounds used in these studies.

Results—Chemical inhibition of the CBF β /RUNX interaction decreases ovarian cancer cell proliferation. Inhibitor treatment leads to an S-phase cell cycle delay, as indicated by an increased percentage of cells in S-phase, and a decreased DNA replication rate. Inhibitor treatment also reduces wound healing and anchorage-independent growth. RNA-Seq on compound-treated cells revealed changes in a small number of genes related to proliferation and epithelial-to-mesenchymal transition. siRNA-mediated knockdown of *INHBA* and *MMP1* – two genes whose expression decreases with compound treatment – slowed DNA replication and impaired wound healing.

Conclusions—Chemical inhibition of the CBF β /RUNX interaction is a viable strategy for the treatment of ovarian cancer.

1. Introduction

Ovarian cancer is the second most common and deadliest gynecologic malignancy[1]. Despite numerous advances in the treatment of other cancers, the standard of care for ovarian cancer has not been significantly modified in the past 20 years, and 5-year survival remains unchanged. Unlike other epithelial cancers, ovarian cancer lacks many of the recurrent mutations that have been successful drug targets[2]. Therefore, novel treatment strategies are urgently needed to combat this disease.

Core-Binding Factor Subunit Beta (CBF β) and a RUNX protein (RUNX1, RUNX2, or RUNX3) form a heterodimeric transcription factor complex that is essential for proper tissue development and differentiation[3]. RUNX proteins contain a DNA-binding domain; however, their affinity for DNA is increased up to 40-fold upon CBF β binding[4]. The two proteins together form the functional transcription factor complex[5]. While CBF β and RUNX proteins are canonically involved in hematopoiesis[6], osteogenesis[7], neurogenesis[8], and gastric development[9], they have an emerging oncogenic role in epithelial cancers[10].

RUNX1, RUNX2, and RUNX3 are overexpressed in a subset of epithelial ovarian cancers[11–13]. Genetic reduction of CBF β , RUNX1, or RUNX2 in ovarian cancer cell lines reduces proliferation and decreases anchorage-independent growth[11, 12, 14, 15]. Upregulation of RUNX3 is associated with carboplatin resistance[13]. However, the downstream mechanisms underlying these effects remain unknown. Additionally, these experiments utilized shRNA-mediated knockdown (KD) to investigate the role of these proteins. While this is a useful tool for validation, it is not a viable therapeutic approach.

Recently, inhibitors of the CBF β RUNX protein-protein interaction have been developed. These compounds bind to CBF β and block its ability to bind RUNX proteins[16]. With these novel tool compounds, we studied both the effects of CBF β inhibition in ovarian cancer and the downstream mechanisms underlying these effects. CBF β inhibition decreases ovarian cancer cell proliferation, migration, and anchorage-independent growth. This phenotype is driven by a small number of gene-expression changes. Knockdown of two differentially expressed genes recapitulates the phenotype observed with compound treatment. Together, these data validate CBF β as a target in ovarian cancer and provide strong rationale for further development of clinically applicable CBF β inhibitors.

2. Materials and Methods

2.1 Cell Culture and Inhibitor Treatment

A2780ip2, PEO1, PEO4, and SKOV3ip1 cell lines were from the Landen lab at the University of Virginia, OVCAR4 cells were from the Slack-Davis lab at the University of Virginia, BEAS-2B cells were a gift from Dr. Thao Dang, and OVCAR8 cells were from ATCC. Culture conditions are detailed in the supplementary methods. Cell line identity was verified by STR profiling. Tool inhibitors were synthesized in the Bushweller lab as previously described[16]. Staurosporine was purchased from Sigma-Aldrich (S5921).

2.2 Cell Viability Assays

Cells (2500–5000 depending on the cell line) were seeded in a 96-well plate, and inhibitors were added the following day. After 3 days, CellTiter-Glo (Promega G7570) was added according to the manufacturer's protocol. For MTT assays, BEAS2-B cells (35,000) were plated in the presence of compounds. Relative cell number was assessed after 3 days by MTT (Invitrogen V13154) per the manufacturer's instructions. Luminescence and absorbance were measured using a PHERAStarPlus microplate reader. Live cell number was determined by trypan blue exclusion. To assess live/dead cell percentage, OVCAR8 cells were treated with compounds for 48 hours. Cells were trypsinized, washed once, and then resuspended in 1x PBS. Fifteen minutes prior to analysis, propidium iodide (Sigma-Aldrich P4864) was added to a final concentration of 1µg/mL and cells were immediately analyzed by flow cytometry.

2.3 Western Blotting

3×10^6 cells were lysed in RIPA buffer with protease inhibitor (Roche 11836170001), and equal volumes of lysate were analyzed by western blotting[17]. Primary antibodies are listed in the supplementary methods.

2.4 EdU labeling and pHH3 IF staining

For EdU labeling, OVCAR8 or OVCAR4 cells were seeded in 4-well chamber slides and treated as described. Cells were pulse labeled with 10µM EdU for 45 minutes, then fixed with 4% paraformaldehyde (PFA). EdU was detected using the Click-iT EdU Kit (Invitrogen C10339) following the manufacturer's protocol. EdU intensity was measured using ImageJ. Immunofluorescence staining was performed as previously described with a primary antibody against phospho-HistoneH3(Ser10) (pHH3) (1:500, Millipore 06-570)[17].

2.5 Cell Cycle Analysis

For unsynchronized cell cycle analysis, OVCAR8 cells were treated with 10µM AI-10-104 for 24 or 48 hours and collected. Following G1/S or G2/M cell cycle arrest, cells were released into media containing DMSO or 5µM AI-10-104, and cells were collected at indicated times. Cells were stained with propidium iodide (Sigma-Aldrich P4864) to measure DNA content as previously described[18]. Cell cycle arrest protocols are provided in the supplementary methods.

2.6 Wound Healing Assays

OVCAR8 cells (250,000) were seeded in 6-well plates. The following morning, cells were washed once with PBS, and culture media was replaced with RPMI supplemented with 2.5% FBS and compounds. After 10 hours, confluent monolayers were scratched with a P1000 pipette tip, and the size of the wound was measured at 0, 14, and 24 hours. For siRNA-mediated knockdown, OVCAR8 cells (80,000) were seeded in 12-well plates. Sixteen hours later, cells were transfected with siRNAs as described in 2.10. Twenty-four hours after transfection, cells were washed once with PBS, and culture media was replaced with RPMI supplemented with 2.5% FBS. After 10 hours, confluent monolayers were scratched with a P1000 pipette tip. Size of the wound was measured at 0 and 24 hours. Images were captured using an EVOS XL Core microscope. Wound size was analyzed using MRI Wound Healing macro for ImageJ (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool).

2.7 Colony Formation in Soft Agar

OVCAR8 cells (2500), OVCAR4 (5000) or SKOV3ip1 cells (1000) were seeded into soft agar in 6-well plates in the presence of compounds. Media on the colonies was changed every 4th day. After 14 days (OVCAR8), 40 days (OVCAR4), or 24 days (SKOV3ip1), colonies were stained with 0.005% crystal violet (Sigma-Aldrich C6158) and imaged using a Gel Doc XR+ (BioRad). For compound addition experiments, OVCAR8 cells (5000) were seeded in soft agar. After 11 days, media on the colonies was changed to media supplemented with either DMSO or 10 μ M AI-14-91. Colonies were treated for 7 days and then stained as above. Colony number was counted using Quantity One 1-D Analysis software (BioRad), or by counting colony number in 4 fields of view per well. Colony size was measured using ImageJ.

2.8 OVCAR8 xenograft

Animal procedures were approved by the University of Virginia Animal Care and Use Committee. Animals were housed on a 12-hour light/dark cycle at 23°C with 30–50% relative humidity. 5.2×10^6 OVCAR8 cells were injected intraperitoneally (i.p.) into 7–8 week old female athymic nude mice (Envigo). Two days later, mice were randomized (n=10/group) and treated i.p. with either vehicle control or 100mg/kg AI-14-91 twice per day for 12 days. AI-14-91 was diluted to 25mg/mL in 0.1M Captisol (Ligand Pharmaceuticals) using the *in situ* formed hydrochloride salt. Compounds were 0.22 μ M sterile filtered prior to use. Mice were weighed twice weekly, and behavior carefully noted, to assess for drug toxicity. Mice were euthanized 29–30 days after the initial xenograft and total tumor weight was measured.

2.9 RNA-Seq and RT-qPCR

OVCAR8 cells were treated for 0, 6 or 24 hours with 5 μ M AI-10-10-4, and RNA was sequenced as previously described[17]. Sequencing data analysis procedures are described in the supplementary methods. Differential gene expression was analyzed using DESeq2[23]. Genes with an adjusted p value of < 0.05 were considered significantly altered. RT-qPCR validation of gene expression changes was performed on an independent set of

RNA. EnrichR analysis was performed by submitting the list of differentially expressed genes (DEGs) to the EnrichR platform[24, 25]. Gene Set Enrichment Analysis (GSEA) was performed using GSEA 3.0 (<http://www.broad.mit.edu/gsea>) [26]. Gene sets were considered enriched when the FDR was <0.05.

RT-qPCR was performed as previously described[17]. Digital Droplet PCR (ddPCR) protocols are detailed in the supplementary methods. Primers were designed using Primer3 (Table S1). Gene expression was normalized to GAPDH or RPL4 using the C_T method.

2.10 siRNA-mediated knockdown

OVCAR8 cells were plated in 12-well plates (80,000) or 4-well chamber slides (35,000). The following morning, cells were transfected with a pool of 4 siRNAs directed at non-targeting, INHBA, or MMP1 to a final concentration of 200 nM using Lipofectamine RNAiMax (Invitrogen 13778) per the manufacturer's protocol. siRNA sequences are provided in Table S2. Knockdown of target genes was confirmed by RT-qPCR. Protein loss following siRNA knockdown was confirmed by immunofluorescence staining for INHBA (1:66, R&D Systems AF338) or MMP1 (1:60, AbCam ab52631) as previously described[17].

2.11 Statistical Methods

Each experiment was completed 3 independent times with n=3-6 technical replicates. Data shown are mean $\pm$ SD of one representative experiment. Data was analyzed using Microsoft Excel and GraphPad PRISM 7.0. Groups were compared using a t-test, one-way ANOVA or two-way ANOVA with Holm-Sidak post-hoc tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ control vs. experimental condition. IC₅₀ values were calculated using the GraphPad PRISM curve fit – log inhibitor vs. normalized response.

3. Results

3.1 Ovarian cancer cell lines are sensitive to treatment with compounds that inhibit CBF β /RUNX binding

A panel of 6 ovarian cancer cell lines was assessed for sensitivity to AI-10-104 and AI-14-91, active inhibitors of CBF β 's binding to RUNX proteins (Figure 1A). All 6 ovarian cancer cell lines had reduced cell number after 3 days of treatment with either active compound (Figure 1B,C), and a range of sensitivities was observed. Treatment with AI-4-88, an inactive control compound with a similar chemical structure (Figure 1A), had minimal effects on cellular proliferation at IC₅₀ concentrations of AI-10-104 and AI-14-91 (Figure 1D). All cell lines express CBF β and at least one RUNX protein (Figure 1E). There was a trend towards a positive correlation between CBF β expression and sensitivity to active inhibitors, though SKOV3ip1 was an exception to this pattern. Given the high levels of heterogeneity within ovarian cancer cell lines, further studies should characterize what molecular features affect cell line sensitivity to compounds. Treatment of OVCAR8 cells with inhibitors did not cause a decrease in the protein levels of CBF β or RUNX proteins (Figure S1A). Additionally, BEAS-2B cells, a normal epithelial cell line, treated with working concentrations of AI-10-104 had no effect on cell number after 3 days of treatment

(Figure S1B). Moving forward, experiments were performed primarily in the OVCAR8 cell line, and key experiments were replicated in the OVCAR4 cell line. These cell lines were chosen because they have high expression of CBF β and have been shown to have a similar gene expression profile to high-grade serous ovarian cancer patient tumors[27].

To determine if the change in cell number was due to reduced growth or increased cell death, OVCAR8 cells were treated with either DMSO or 7 μ M AI-10-104, and the number of live cells was measured by trypan blue exclusion after 1, 3, and 5 days. Inhibitor treatment significantly reduced the number of live cells (Figure 1F). The 7 μ M dose was used because it is the average IC₅₀ for the OVCAR8 cell line. To confirm that the number of dead cells did not increase with compound treatment, OVCAR8 cells were treated with high doses of compounds for 48 hours, and the percentage of dead cells was measured by flow cytometry. Treatment with 20 μ M AI-4-88, 10 μ M AI-101-04, or 20 μ M AI-14-91 caused minimal cell death, while causing significant reductions in total cell number (Figure 1G,H). There was a statistically significant increase in cell death with 20 μ M AI-14-91 treatment; however, the absolute percentage of dead cells increased from an average of 0.6% in the DMSO treatment to an average of 1.8% in the AI-14-91 treatment, which we do not believe has biological significance.

3.2 AI-10-104 treatment reduces mitotic index and decreases EdU incorporation

Due to the reduced cell number following compound treatment, we investigated the mitotic index of OVCAR8 cells. Cells were treated with 10 μ M AI-10-104 for 24 or 48 hours and proliferation was measured by pHH3 staining, which marks cells in late G2 and mitosis[28]. Treatment with AI-10-104 significantly decreased the percentage of cells staining positive for pHH3 (Figure 2A,B), indicating a slowed proliferative rate.

Due to the reduction in cell proliferation and mitotic index following tool compound treatment, we investigated the cell cycle profile of OVCAR8 cells exposed to AI-10-104 by propidium iodide staining. Asynchronously dividing OVCAR8 cells treated with AI-10-104 for 24 or 48 hours did not show a G1 arrest, as has been previously described following RUNX1 knockdown in ovarian cancer[11]. However, there was a significant increase in the number of cells in S-phase (Figure 2C, S2A).

To more accurately assess S-phase kinetics after tool compound treatment, OVCAR8 cells were treated with 10 μ M AI-10-104 for 24 or 48 hours. Cells were pulse labeled with EdU immediately prior to fixation. The proportion of cells incorporating EdU, a thymidine analog that marks active DNA replication, was significantly decreased after both 24 and 48 hours of compound treatment (Figure 2D,F). In addition to the decrease in the proportion of cells incorporating EdU, the intensity of each positive nucleus was significantly reduced, indicating a decreased rate of DNA replication (Figure 2G). These findings were replicated in the OVCAR4 cell line, which was treated with 10 μ M AI-10-104 for 48 or 72 hours. The proportion of OVCAR4 cells incorporating EdU was significantly decreased after 72 hours, with a strong trend towards a decrease at 48 hours ($p=0.08$) (Figure 2E,H). OVCAR4 cells were treated for longer than OVCAR8, because their doubling time is substantially longer.

3.3 AI-10-104 treatment slows S-phase progression in synchronized cells

To probe the apparent dichotomy between propidium iodide staining, indicating compound treatment increased the percentage of cells in S-phase, and EdU incorporation results, indicating the opposite, the cell cycle kinetics of synchronized cells was examined. OVCAR8 cells were arrested at the G1/S boundary using a thymidine double block. Cells were then released from arrest into DMSO or 5 μ M AI-10-104. A low dose of compound was used to ensure that the observed effects were not influenced by cellular toxicity. Cell cycle progression was measured by propidium iodide staining at multiple time points over the next 30 hours.

As compound treated cells progressed through the cell cycle, their rate of S-phase completion was slowed relative to DMSO treated cells. A mild reduction in the rate of S-phase completion was observed at 6 hours – the first S-phase with compound treatment (Figure 2I). A more profound decrease in the rate of S-phase completion was observed 24 hours after release from the G1/S boundary. Comparable results were obtained when cells were arrested at the G2/M boundary by nocodazole and released into DMSO or AI-10-104 (Figure S2B).

These data indicate that while compound treated cells can progress through the cell cycle, the rate at which they do so is diminished due to a slow progression through S-phase. This S-phase delay leads to an increased percentage of cells in S-phase as measured by total DNA content. The apparent decrease in the proportion of cells in S-phase as measured by percent of EdU+ cells is likely secondary to the decreased rate of incorporation. Cells with slow DNA replication velocity would both spend longer in S-phase and be less likely to incorporate EdU above the detection threshold.

3.4 AI-10-104 and AI-14-91 treatment impair wound healing and colony formation in soft agar

As CBF β and RUNX proteins have been shown to modulate migration in ovarian and other epithelial cancers, we investigated the ability of tool inhibitors of the CBF β /RUNX interaction to impair cell mobility, using a wound healing assay. Confluent monolayers of OVCAR8 cells were scratched and wound size was measured at 0, 14, and 24 hours. Treatment with AI-10-104 and AI-14-91 significantly impaired wound healing (Figure 3A,B).

To assess the ability of tool compounds to decrease anchorage-independent growth, OVCAR8 cells were plated in soft agar in the presence of either AI-10-104 or AI-14-91, and the number of colonies was counted after 14 days. Inhibitor treatment significantly reduced the number of colonies, from an average of 176 colonies per well in the control to an average of 12 colonies per well across all compound treatments (Figure 3C, S3A). Treatment with AI-4-88, the inactive control compound, did not appear to alter colony formation, indicating that this dramatic effect likely results from CBF β /RUNX inhibition specifically. To ensure that this reduction in colony formation was not specific to the OVCAR8 cell line, colony formation in the presence of compounds was investigated in SKOV3ip1 and OVCAR4 cell

lines. Similar significant reductions in colony formation were observed (Figure 3D,E, S3B,C).

Prior experiments assessed the ability of tool compounds to inhibit initial colony formation as well as subsequent colony growth. Thus, we assessed the ability of the tool compounds to inhibit the growth of already-formed colonies. OVCAR8 cells were seeded in soft agar, and incubated for 11 days. Colonies were then treated with DMSO or 10 μ M AI-14-91 for 7 days. While the number of colonies did not change between treatments, the size of the inhibitor-treated colonies was significantly reduced (Figure 3F-H), indicating that tool compounds can decrease the growth rate of established colonies in addition to inhibiting the generation of new colonies.

To extend these results to an *in vivo* model of anchorage-independent growth, the effects of AI-14-91 were tested in a xenograft model of ovarian cancer. AI-10-104 is not suitable for use in animals[16]. Athymic nude mice were injected i.p. with OVCAR8 cells. Two days later, mice were randomized and treated with vehicle control or AI-14-91 at 100mg/kg twice per day for 12 days. Thirty days following xenografting, total tumor weight from the peritoneal cavity was measured. There was no significant change in total tumor weight; however, this is likely due to the limited half-life and low potency of AI-14-91 (Figure S4A). Pharmacokinetic data indicates that CBF β was likely only inhibited for ~2 hours following each injection[16]. Treatment with AI-14-91 did not cause any overt toxicity or weight loss (Figure S4B).

3.5 Inhibition of CBF β with AI-10-104 alters the transcription of a small network of genes

As CBF β constitutes an integral component of the CBF β /RUNX transcription factor complex, we hypothesized that CBF β controlled the expression of a network of genes regulating proliferation, migration, and anchorage-independent growth. To assess the network of genes controlled by CBF β in ovarian cancer, we performed RNA-Seq on OVCAR8 cells treated with 5 μ M AI-10-104 for 0, 6 or 24 hours. We chose a short time course to identify the most proximal effects of CBF β /RUNX inhibition and used a low dose of compound to reduce the chance of any confounding toxicity. Analysis of differential gene expression using DESeq2 revealed a small set of genes altered by compound treatment (Figure 4A,B, Table S2). Alterations in a panel of DEGs, including INHBA, MMP1, COL7A1, and IGFN1 were confirmed by RT-qPCR (Figure 4C). Additionally, these changes in gene expression were replicated in the OVCAR4 cell line indicating these findings are not unique to the OVCAR8 cells (Figure 4D).

DEGs were analyzed for transcription factor enrichment using EnrichR. Five transcription factor signatures from the ChEA 2016 data set were significantly enriched, including SMAD2 and SMAD3 (Figure 4E). GSEA revealed enrichment of seven hallmark gene signatures, including signatures for the “Mitotic Spindle” and “Epithelial-to-Mesenchymal Transition” (EMT)(Figure 4F, S5A,B).

3.6 siRNA-mediated knockdown of differentially expressed genes decreases EdU incorporation and impairs wound healing

The differentially expressed genes *INHBA* and *MMP1* were chosen for further analysis, as they are likely candidates to explain the phenotypes observed following tool compound treatment. These genes are included in the GSEA EMT gene list, are SMAD2/3 target genes, and have been putatively shown to be CBF β or RUNX regulated in cancer [11, 12, 29, 30]. Additionally, INBHA is the monomeric subunit of Activin A, which has been shown to promote ovarian oncogenesis by stimulating the migration of fallopian tube secretory epithelial cells [31].

To assess the influence of the above genes on cell cycle progression, OVCAR8 cells were seeded in 4-well chamber slides and transfected with a pool of 4 non-targeting siRNAs or a pool of 4 siRNAs targeting INHBA or MMP1. Three days after transfection, cells were pulse labeled with EdU. siRNA-mediated knockdown of MMP1 significantly reduced the proportion of cells incorporating EdU, as observed following tool compound treatment (Figure 5A,B). siRNA-mediated knockdown of INBHA showed a trend towards decreased EdU incorporation; however, it did not meet statistical significance. siRNA knockdown was confirmed by RT-qPCR (Figure 5G). To confirm that RNA knockdown was accompanied by a corresponding decrease in protein expression, OVCAR8 cells were transfected with non-targeting siRNAs or siRNAs targeting INHBA or MMP1. Protein levels of INHBA and MMP1, as measured by immunofluorescence, were decreased after siRNA targeting, indicating that RNA knockdown corresponds to decreased protein expression (Figure S6A,B).

To assess the role of these genes in migration, OVCAR8 cells were transfected with a pool of 4 non-targeting siRNAs or a pool of 4 siRNAs targeting INHBA or MMP1. Confluent monolayers were scratched, and wound healing was assessed after 24 hours (Figure 5C,D,H). siRNA targeting of either gene significantly decreased wound healing, consistent with the phenotype observed following tool CBF β /RUNX inhibitor treatment.

3.7 Dual knockdown of INHBA and MMP1 enhances wound healing inhibition

As *INHBA* and *MMP1* are both included in the hallmark EMT gene list, we assessed if dual targeting of these genes would have combinatorial effects on wound healing. To measure the relative contribution of each gene to this process, genes were targeted independently or in combination, and the effect on wound healing was measured.

OVCAR8 cells were transfected with a total of 20pmol of siRNAs targeting either a single gene or both genes together. When targeting individual genes, half of the siRNA was non-targeting. As expected, siRNA-mediated knockdown of each gene independently decreased wound healing, even when the amount of siRNA transfected was reduced by 50%. Knockdown of both genes together yielded an even greater reduction in wound healing (Figure 5E,F,I). These results indicate that each gene has a non-redundant contribution to wound healing, suggesting that CBF β may control multiple mediators of EMT.

4. Discussion

In this study, we demonstrate that CBF β inhibition leads to decreased growth, migration, and anchorage independent growth of ovarian cancer cells in multiple cell lines. Additionally, CBF β /RUNX inhibition causes a decrease in INHBA and MMP1 mRNA, and knockdown of these genes recapitulates the effects of inhibitor treatment. This work further validates the oncogenic role CBF β plays in ovarian cancer and uses a clinically applicable small molecule inhibition strategy.

These results particularly emphasize the role of CBF β in migration and anchorage-independent growth. Doses of inhibitors needed to significantly impair wound healing or colony formation were much lower than those required for growth inhibition. This result is of particular importance in ovarian cancer as anchorage-independent growth and migration are strong models of metastasis. The majority of women with ovarian cancer are diagnosed with metastatic disease, and these metastases are a primary driver of ovarian cancer morbidity and mortality[2]. CBF β /RUNX inhibition may be uniquely useful as an adjuvant therapy to inhibit further development of metastatic lesions.

While AI-14-91 had significant effects on cells grown in culture, it did not inhibit tumor growth in an *in vivo* xenograft model of ovarian cancer. AI-14-91, while a useful chemical probe, has several significant limitations for *in vivo* use. AI-14-91 has low potency and a short half-life. These two limitations combined lead to an estimated ~2 hours of CBF β inhibition per dose. Thus, even with twice daily dosing, the protein-protein interaction between CBF β and RUNX was likely only inhibited for ~4 hours per day[16]. Compounds with increased potency should be evaluated for their success in this model. In addition to ongoing structure-activity-relationship work, alternate strategies could be employed to increase the potency of current compounds, such as generating a PROTAC (proteolysis targeting chimera) compound. PROTACs are bivalent compounds which bind to their target protein and an E3 ubiquitin ligase, leading to target protein degradation[32]. This irreversible inhibition of CBF β would combine the beneficial features seen with chemical inhibition, as well as likely recapitulate data generated using shRNAs.

While we were surprised at the relatively small number of DEGs following compound treatment, this result is not unprecedented. Analysis of gene expression changes after treatment with Kartogenin, a CBF β /FilaminA inhibitor, revealed only 39 DEGs, and siRNA-mediated knockdown of CBF β in a metastatic breast cancer cell line yielded 161 DEGs, many fewer than would be predicted following knockdown of a transcription factor[29, 33]. While the specific genes altered in these studies do not have large overlap, this is likely due to the differing cell lineages. However, more interestingly, perturbation of CBF β , whether chemical or genetic, revealed recurrent alterations in EMT and extracellular matrix formation. Kartogenin was identified during a screen for compounds that promote chondrogenesis, and Kartogenin treatment increases collagen production. GO analysis of genes altered by siRNA targeting of CBF β in metastatic breast cancer revealed enrichment of the “extracellular matrix organization” and “regulation of cell adhesion biological processes”, mimicking our results. These data externally validate our findings demonstrating the contribution of CBF β specifically to EMT.

The relatively small changes in gene expression across all 3 experiments likely indicate that CBF β controls a very specific and narrow transcriptional network. It is also possible that there is an undefined non-transcriptional function of CBF β . CBF β has reported protein-protein interactions with several components of the cytoskeleton, including Collagen Alpha-1(VII) Chain (*COL7A1*), whose transcript levels were decreased after CBF β /RUNX inhibitor treatment. Additionally, CBF β has been identified at the midbody during cytokinesis, though its function there is not well defined[34]. These interactions may provide additional mechanistic insight into the role of CBF β as a regulator of cellular proliferation and migration. Future studies should investigate alterations in protein-protein interactions following inhibitor treatment. These experiments may provide insight into these potential non-transcriptional effects of CBF β .

In summary, CBF β is a druggable target in ovarian cancer, and chemical inhibition of its interaction with RUNX proteins decreases proliferation, migration, and anchorage-independent growth. These effects are partially explained by alterations in gene expression. Future work identifying higher potency compounds and extending these findings to an *in vivo* model will both help improve ovarian cancer treatment and provide novel therapeutic strategies for a difficult to target disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CBF β inhibition decreases ovarian cancer proliferation and causes an S-phase delay
- CBF β inhibition decreases wound healing and anchorage-independent growth
- INHBA and MMP1 decrease following CBF β inhibition
- Knockdown of INHBA or MMP1 mimics the effects of CBF β inhibitor treatment

Figure 1.

Effects of inhibitors on cell growth. A. Table displaying IC₅₀ values from a FRET-based assay of CBF β /Runt domain binding and structures of tool compounds. B. Relative live cell number measured using CellTiter-Glo after 3 days of AI-10-104 treatment (n=3/dose). C. Relative live cell number measured using CellTiter-Glo after 3 days of AI-14-91 treatment (n=3/dose). D. Relative live cell number measured using CellTiter-Glo after 3 days of AI-4-88 (n=3/dose). E. Western blot of 6 ovarian cancer cell lines for CBF β , RUNX1, RUNX2, and RUNX3. One representative HSP90 blot is shown as a loading control. F. OVCAR8 cells were treated with DMSO or 7 μ M AI-10-104. Number of live cells was

counted by trypan blue exclusion at the indicated time. G. Relative OVCAR8 cell number after treatment with compounds for 48 hours. H. Percentage of live and dead OVCAR8 cells after compound treatment for 48 hours. Live/Dead analysis was performed by propidium iodide exclusion and measured by Flow Cytometry. ** $p < 0.01$, *** $p < 0.001$, by one-way ANOVA (G,H) or two-way repeated measures ANOVA (F) with Holm-Sidak post-hoc tests.

Figure 2.

Effects of inhibitors on cell cycle kinetics. A. OVCAR8 cells were treated with 10μM AI-10-104 for 0, 24 or 48 hours and then stained with anti-pHH3(Ser10). Representative images are displayed. B. Quantification of pHH3 staining (n=4/time). C. Percentage of cells in each phase of the cell cycle measured by propidium iodide staining of DNA content and analyzed by Flow Cytometry (n=3/time). D. OVCAR8 cells were treated with 10μM AI-10-104 for 0, 24, or 48 hours then pulsed with EdU immediately prior to fixation. EdU incorporation was measured using Click-iT™ Imaging Kit. Representative images are shown. E. OVCAR4 cells were treated with 10μM AI-10-104 for 0, 48 or 72 hours then pulsed with EdU

immediately prior to fixation. EdU incorporation was measured using Click-iT™ Imaging Kit. Representative images are shown. F. Quantification of EdU+ OVCAR8 cells relative to total nuclei (n=4/time). G. Quantification of mean EdU intensity of each positive OVCAR8 nucleus (n=4/time). H. Quantification of EdU+ OVCAR4 cells relative to total nuclei (n=4/time). I. OVCAR8 cells were arrested at the G1/S boundary and released into DMSO or 5μM AI-10-104. DNA content was measured by propidium iodide staining at the indicated times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by one-way ANOVA with Holm-Sidak post-hoc tests.

Figure 3.

Effects of inhibitors on wound healing and colony formation. A-B. Confluent monolayers of OVCAR8 cells were scratched with a pipette tip. Size of the wound was imaged at 0, 14, and 24 hours. A. Representative images of wounds. Wound is highlighted in green. B. Quantification of wound closure (n=3 wounds/dose). C-E. Colony formation in soft agar of ovarian cancer cells treated with compounds. C. Total number of OVCAR8 colonies per well (n=6 wells/dose). D. Number of OVCAR4 colonies per 4 fields of view (n=6 wells/dose). E. Total number of SKOV3ip1 colonies per well (n=6 wells/dose). F-H OVCAR8 cells were seeded in soft agar. After 11 days, colonies were treated with DMSO or 10μM AI-14-91 for

7 days. F. Total number of colonies following compound treatment (n=6/dose). G. Average colony size (n=6/dose) H. Representative images of colonies following 7 days of compound treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by t-test (F,G), one-way ANOVA (C,D,E), or two-way repeated measure ANOVA (B) with Holm-Sidak post-hoc tests.

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Figure 4. Effects of inhibitors on gene expression. A. Venn diagram of differentially expressed genes (DEGs) following 6 or 24 hours of treatment with 5µM AI-10-104 measured by RNA-Seq. Differential gene expression was analyzed using DESeq2 with a significance cutoff of padj<0.05 (n=3 samples/condition). B. Volcano plot of DEGs. C-D. Confirmation of DEGs in OVCAR8 and OVCAR4 cells treated with AI-10-104 for the indicated time. C. RT-qPCR analysis of gene expression in OVCAR8 cells. Expression of each gene is normalized to GAPDH (n=3 samples/dose). D. ddPCR (INHBA, MMP1, IGFBP1) or RT-qPCR (COL7A1) analysis of gene expression in OVCAR4 cells. Expression of each gene is normalized to

GAPDH (n=3 samples/dose). E. Significantly enriched promoter signatures in DEGs assessed from the ChEA 2016 database using EnrichR. F. GSEA enrichment plots for 2 of the hallmark gene signatures enriched in drug-treated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by t-test (D) or one-way ANOVA (C) with Holm-Sidak post-hoc tests.

Figure 5.

siRNA mediated knockdown of compound-altered genes mimics compound treatment. A-B. siRNA mediated KD of INHBA or MMP1 was performed on OVCAR8 cells in 4-well chamber slides. Cells were pulsed with EdU for 45 minutes immediately before fixation. A. Representative images of EdU incorporation following siRNA-mediated KD. B. Quantification of ratio of EdU+ nuclei/total nuclei (n=4 chambers/siRNA). C-D. siRNA-mediated KD of INHBA or MMP1 was performed. Confluent monolayers of KD cells were scratched with a pipette tip, and percent wound closure was assessed at 24 hours. C. Representative images of wounds at 0 and 24 hours. Wounds are highlighted in green. D.

Quantification of wound closure after 24 hours (n=3 wounds/siRNA). E-F. Compound-affected genes were knocked down alone or in combination, and effects on wound healing were assessed. E. Representative images of wounds following single or combined siRNA-mediated KD. Wounds are highlighted in green. F. Percent wound closure after 24 hours following single or combined KD (n=3 wounds/siRNA). G. Knockdown as measured by RT-qPCR of genes from Figure 5A,B. H. Knockdown as measured by RT-qPCR of genes from Figure 5C,D. I. Knockdown as measured by RT-qPCR of genes from Figure 5E,F. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by t-test (G,H) one-way ANOVA (B,D,F,I) with Holm-Sidak post-hoc tests.