New 1,1-diarylethylene FOXM1 inhibitors potently reduce intracellular FOXM1 and suppress high-grade serous ovarian carcinoma cell viability

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Research Article

Keywords:

Posted Date: January 25th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1281554/v1>

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Abstract

As an oncogenic transcription factor highly upregulated in numerous cancer types and responsible for promoting multiple hallmarks of the disease, Forkhead Box M1 (FOXM1) is a promising target for treatment against high-grade ovarian serous carcinoma (HGSC), a lethal and aggressive cancer that overexpresses FOXM1 and its transcriptional pathway. Several FOXM1 inhibitors have been established, but none have advanced to clinical trials. In this study, we report that our recently developed 1,1-diarylethylene FOXM1 inhibitors (NB compounds) potently and selectively inhibit HGSC cell viability, more so than a panel of other reported FOXM1 inhibitors. The NB compounds decreased FOXM1 expression through proteasomal degradation, which resulted in decreased expression of its downstream target cyclin B1 (CCNB1), without affecting other FOX family members. Moreover, the NB compounds exhibited robust anti-cancer effects by promoting apoptosis, suppressing colony formation more potently than other FOXM1 inhibitors, and synergizing with carboplatin to inhibit the viability of HGSC cells. Our data demonstrate that the NB compounds are promising FOXM1 inhibitors that may serve as a novel therapeutic strategy for HGSC and other cancers whose aggressive cancer biology is driven by FOXM1 and its oncogenic transcriptional pathway.

Introduction

High-grade serous ovarian carcinoma (HGSC) comprises over 60% of ovarian cancers¹ and accounts for up to 90% of ovarian cancer-related deaths². Hence, effective treatments for HGSC, especially for relapsed disease, are needed. Forkhead Box M1 (FOXM1) is a master transcriptional regulator of cell cycle and mitotic progression and has numerous oncogenic functions in multiple cancers, including ovarian cancer³⁻⁸. We have previously shown that FOXM1 is widely overexpressed in HGSC⁹ and other cancers¹⁰, driven by multiple molecular mechanisms such as copy-number gains and disruption in p53 and Rb signaling⁸⁻¹⁰. Mounting evidence supports the multifunctional oncogenic role of FOXM1 in promoting ovarian cancer cell proliferation, invasion and metastasis, chemotherapy resistance, cancer cell stemness, genomic instability, and altered cellular metabolism⁸. The FOXM1 transcriptional pathway is aberrantly activated in over 85% of high-grade serous ovarian carcinoma (HGSC) cases^{9,11}, second only to *TP53* mutations (100% of HGSC cases¹²), suggesting that FOXM1 may be a promising target for HGSC therapy.

Recognizing the oncogenic functions of FOXM1 prompted the development of small molecule inhibitors (SMIs) targeting FOXM1 in pursuit of clinical translatability. Among FOXM1 inhibitors, thiostrepton and Forkhead Domain Inhibitor 6 (FDI-6) are the most extensively studied. Thiostrepton is a thiazole antibiotic that promotes FOXM1 degradation¹³⁻¹⁵, but its exact mechanism of action (MOA) is presently debated¹⁵⁻¹⁷. FDI-6 was identified through a high-throughput screen (HTS) for SMIs that disrupt the interaction between the DNA binding domain of FOXM1 and its consensus promoter sequence¹⁸. However, as other forkhead box (FOX) family members share this sequence^{19,20}, this approach may limit the selectivity of FDI-6 and disregards FOXM1 functions related to promoter binding to non-consensus sequences and

super-enhancer regions²¹⁻²⁴. Thus, inhibiting the DNA binding domain function alone may not be sufficient to prevent these other critical interactions. Other reported FOXM1 inhibitors include monensin²⁵, N-phenylphenanthren-9-amine²⁶, and RCM-1²⁷, all of which are much less characterized and appear to work by various mechanisms with less defined efficacy and selectivity.

We recently developed novel FOXM1 inhibitors based on a 1,1-diarylethylene core structure: NB-55 (monoamine), NB-73 (diamine methiodide salt), and NB-115 (diamine methiodide salt)²⁸. NB-55, NB-73, and NB-115 (NB compounds) were found to promote the degradation of FOXM1 and inhibit breast cancer cell proliferation at nanomolar concentrations²⁸. These NB compounds induced cell cycle arrest and apoptosis in triple-negative breast cancer (TNBC) cells and reduced TNBC xenograft growth in mice²⁸. They also decreased migration and invasion phenotypes and suppressed epithelial-to-mesenchymal transition (EMT) and metastasis-related gene expression in TNBC cells²⁹.

HGSC has a highly similar molecular profile to TNBC, including *TP53* mutations, Rb pathway dysregulation, homologous recombination DNA repair deficiency, and extensive somatic copy number alterations³⁰. Moreover, both TNBC and HGSC show similar functional *FOXM1* copy number gains, which translate to increased FOXM1 gene and protein expression¹⁰. Therefore, we sought to investigate the anti-cancer effects of these novel FOXM1 inhibitors in HGSC. Here, we elucidate the capacity of the NB compounds to promote the death of HGSC cells, explore the underlying mechanisms of their actions, and provide a systemic and thorough characterization of the potency and efficacy of the NB compounds in comparison to reported FOXM1 inhibitors.

Materials And Methods

Chemical Compounds

Full details on the preparation and spectroscopic characterization of the NB compounds have been previously described²⁸. NB-73, NB-115, Thiostrepton (Sigma-Aldrich #598226), FDI-6 (Sigma-Aldrich #SML1392), RCM-1 (R&D Systems #6845), N-phenylphenanthren-9-amine (Sigma-Aldrich #761966), proteasome inhibitor MG132 (Sigma-Aldrich #474790), general caspase inhibitor Q-VD-OPH (R&D Systems #OPH001), and olaparib (Selleck Chemicals #S1060) were dissolved in DMSO. NB-55 and monensin (R&D Systems #5223) were dissolved in ethanol. Carboplatin (Sigma-Aldrich #C2538) was dissolved in water. All compounds were stored at -20 °C.

Cell Culture and Reagents

CAOV3 and OVCAR5 cell lines were a gift from Dr. Anirban Mitra (Indiana University). CAOV3, OVCAR5, OVCAR8 (National Cancer Institute Division of Cancer Treatment and Diagnosis Cell Line Repository), COV318 (Sigma), and FUOV1 (MD Anderson) cell lines were cultured in DMEM (Corning) supplemented

with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (pen-strep, Gibco). OVCAR4 (National Cancer Institute Division of Cancer Treatment and Diagnosis Cell Line Repository) and OVSAHO (Japanese Collection of Research Bioresources Cell Bank) cell lines were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS and 1% pen-strep. The human immortalized fallopian tube epithelial (FTE) cell line FT282, a gift from Dr. Ronny Drapkin (University of Pennsylvania)³¹, was used to generate the clonal cell line FT282 C11³². The FT282 C11 cell line was cultured in DMEM and Ham's F12, 50/50 mix (Corning) supplemented with 10% FBS and 1% pen-strep. All cell lines were maintained at 37 °C in a humidified incubator with 5% CO₂.

Stocks of all cell lines were authenticated using short tandem repeat analysis at the DNA Services Facility, University of Illinois at Chicago. All cell line stocks were confirmed to be mycoplasma-free by PCR analysis using Mycofind™ Mycoplasma PCR Detection Kit (Clongen Laboratories, LLC).

Western blotting

Whole cell protein extracts were prepared using radio-immunoprecipitation assay (RIPA) buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Briefly, cells were lysed for 15 minutes in RIPA buffer and then sonicated, and the resulting extracts were centrifuged at 4 °C for 10 minutes at 16,100 x *g* to remove cell debris. Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Fisher Scientific).

Equal amounts of protein (20 µg) were separated on 7.5% Mini-Protein® TGX™ precast protein gels (Bio-Rad) and transferred to 0.45 µm polyvinylidene difluoride (PVDF) transfer membranes (MilliporeSigma). Membranes were stained with Ponceau S to confirm efficient transfer and equal loading. The membranes were then de-stained with Tris-buffered saline Tween-20 (TBS-T), blocked with 5% nonfat dry milk in TBS-T for 1 hour at room temperature, and incubated with primary antibodies in 5% bovine serum albumin (BSA) in TBS-T at 4 °C overnight. Primary antibodies were used against FOXM1 (Cell Signaling Technology #5436, 1:1,000), p-FOXM1 (Thr600) (Cell Signaling Technology #14655, 1:2,000), CCNB1 (Cell Signaling Technology #4138, 1:2,000), FOXA1 (Cell Signaling Technology #53528), FOXO3A (Cell Signaling Technology #12829, 1:10,000), Ubiquitin (Cell Signaling Technology #14049, 1:20,000), and HSP70 (Cell Signaling Technology #4872, 1:1,000). When appropriate, the membranes were then incubated in goat anti-rabbit secondary antibody (Cell Signaling Technology #7074, 1:5,000) in 5% milk in TBS-T for 1 hour at room temperature. SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Fisher Scientific) was used for protein detection. Ultra blue X-ray films (Light Labs) were applied to the blots and developed in the film processor. Quantification of protein expression was performed using Fiji software³³.

Apoptosis Assays

Cells were seeded into 6-well plates and treated with the designated compound(s) until the desired time point. The spent medium containing floating cells as well as trypsinized cells from the wells were collected, centrifuged at 500 x *g* to form a pellet, and washed with PBS. Cells were then prepared for apoptosis analysis using the Muse[®] Caspase-3/7 Kit (Luminex Corporation) according to the manufacturer's instructions. Cellular apoptosis was analyzed by flow cytometry using the Guava[®] Muse[®] Cell Analyzer (Luminex Corporation).

All assays were performed in three independent trials and analyzed using GraphPad Prism 7 software.

Colony Formation Assays

For two-dimensional, anchorage-dependent colony formation assays, cells in single-cell suspension were seeded in triplicate wells of 6-well plates, treated with the designated compound(s), and allowed to form colonies for 7-14 days, depending on the cell line. Following incubation, cells were fixed in methanol, stained with crystal violet, washed with water, and air-dried overnight. Pictures of the wells were captured and colonies were counted using Count and Plot Histograms of Colony Size (countPHICS) software³⁴, a macro written for ImageJ. The threshold for colony size was automatically determined by countPHICS.

For three-dimensional, anchorage-independent (soft agar) colony formation assays, cells in single-cell suspension were seeded into a 0.4% SeaPlaque[™] Agarose (Lonza Bioscience) liquid solution and placed on top of a solidified layer of 0.8% SeaPlaque[™] Agarose solution in triplicate wells of 6-well plates. The top layer with cells was allowed to solidify at room temperature for at least 30 minutes. Cells were then treated with the designated compound(s) and allowed to form colonies for 14 days. Following incubation, cells were stained with crystal violet and washed with water. Pictures of the wells were taken, and colonies were counted using countPHICS³⁴. The threshold for colony size was automatically determined by countPHICS.

Cell Viability Assays

Cells were seeded into 96-well plates and treated in serial dilutions with the designated compound(s) for 72 hours. Medium was then removed from the wells, and the cells were frozen at -80 °C. Cell viability was analyzed using the CyQUANT[™] Cell Proliferation Assay, for cells in culture (Invitrogen) according to manufacturer's instructions. Fluorescence intensity was measured using POLARstar OPTIMA microplate multi-detection plate reader (BMG LabTech) with settings according to manufacturer's instructions.

For cell viability assays examining synergistic interactions, the same method was followed as described above. Concentrations of each compound were decided based on the diagonal method to measure drug

synergy³⁵. Synergy was analyzed using CompuSyn software (ComboSyn, Inc.).

All assays were performed in three independent trials with three technical replicates per independent trial and analyzed using GraphPad Prism 7 software.

Statistical Analyses

Statistics were calculated using Student's *t*-test, analysis of variance (ANOVA), or 2-way ANOVA with multiple comparisons, as appropriate, using GraphPad Prism 7 software. Significance was designated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

Results

NB compounds decrease FOXM1 protein expression levels in HGSC cells

To assess whether the NB compounds function as FOXM1 inhibitors in HGSC cells, HGSC cell lines CAOV3 and OVCAR4 were treated with the NB compounds for 6 hours, 24 hours, and 48 hours and examined for FOXM1, p-FOXM1 (Thr600), Cyclin B1 (CCNB1), FOXA1, and FOXO3A protein expression. Phosphorylation of FOXM1c at the threonine 600 residue (threonine 585 residue on FOXM1b) is widely considered critical for FOXM1 function^{36,37}. Once activated, FOXM1 promotes the transcription of numerous cell cycle genes, including *CCNB1*³⁸⁻⁴⁰. FOXA1 is a FOX family member suggested to have oncogenic functions in epithelial ovarian cancer cells^{41,42}, and FOXO3A is a FOX family member with tumor suppressor functions⁴³. Like FOXM1, both FOXA1 and FOXO3A are strong transcriptional regulators that recognize the canonical RYAAAYA forkhead binding motif and therefore served as FOX family control proteins.

NB-55 decreased FOXM1, p-FOXM1, and CCNB1 protein expression at 6, 7, and 8 μ M concentrations in CAOV3 cells (**Figure 1A**). However, there was a narrow window of activity, with almost all CAOV3 cells dying by 24 hours when treated with 8 μ M NB-55 and by 48 hours with 7 μ M NB-55 treatment. Interestingly, at six hours treatment, p-FOXM1 and CCNB1 showed reduced expression while FOXM1 levels remained stable. This observation suggests that the NB compounds may also promote the dephosphorylation of FOXM1, which would be a novel MOA for the NB compounds, in addition to FOXM1 degradation. FOXA1 increased in expression at 48 hours, and FOXO3A temporarily increased in expression at 24 hours. NB-55 treatment of OVCAR4 cells (**Figure 1B**) demonstrated a similar pattern for FOXM1, p-FOXM1, and CCNB1 expression, though CCNB1 protein expression was not detectable by western blot at six hours. There was a slight decrease in FOXA1 at 24 hours, which appeared to rebound at 48 hours, and FOXO3A did not appear to change considerably.

NB-73 induced a prominent decrease in FOXM1, p-FOXM1, and CCNB1 expression in CAOV3 cells at 1 and 2.5 μ M concentrations in a time- and dose-dependent manner (**Figure 1C**). FOXA1 expression

increased at 48 hours NB-73 treatment, and FOXO3A expression was largely unaffected by NB-73 treatment. In OVCAR4 cells (**Figure 1D**), FOXM1, p-FOXM1, and CCNB1 demonstrated a similar time- and dose-dependent decrease in expression at 24- and 48-hour treatment. FOXA1 expression decreased at 24 and 48 hours, and FOXO3A expression was largely unaffected by NB-73 treatment.

NB-115 also induced a prominent decrease in FOXM1, p-FOXM1, and CCNB1 expression at 1 and 2.5 μ M concentrations in a time- and dose-dependent manner in CAOV3 cells (**Figure 2A**). FOXA1 expression increased at both 24 and 48 hours, and FOXO3A expression slightly increased at 24 hours. In OVCAR4 cells (**Figure 2B**), FOXM1, p-FOXM1, and CCNB1 demonstrated a similar time- and dose-dependent decrease in expression at 24- and 48-hour treatment. FOXA1 expression increased at 24 hours and then decreased at 48 hours. FOXO3A expression was largely unaffected by NB-115 treatment.

For comparison, we also examined changes in protein expression in CAOV3 and OVCAR4 cells treated with FDI-6 or thiostrepton, two of the most established FOXM1 inhibitors. Given the reported mechanisms of thiostrepton¹⁵⁻¹⁷ and FDI-6¹⁸, we anticipated that thiostrepton would promote degradation of overall FOXM1 protein while FDI-6 would inhibit downstream CCNB1 expression without promoting the degradation of FOXM1 or p-FOXM1 protein. In CAOV3 cells (**Figure 2C**), FDI-6 did not induce a reduction of FOXM1 or p-FOXM1 expression at 24 hours and 48 hours, while CCNB1 expression decreased equally at both 24 and 48 hours. FOXA1 expression decreased at 48 hours, while FOXO3A expression did not change. Thiostrepton, on the other hand, induced a decrease of FOXM1 and p-FOXM1 expression at 24 hours and CCNB1 expression at 48 hours in a time-dependent manner. FOXA1 expression decreased at 48 hours, and FOXO3A expression temporarily increased 4-fold at 24 hours. In OVCAR4 cells (**Figure 2D**), FDI-6 did not induce a reduction of FOXM1 or p-FOXM1 expression at 24 and 48 hours, while CCNB1 expression transiently decreased at 24 hours. FOXA1 was largely unchanged, and FOXO3A expression increased slightly at 24 hours. Thiostrepton induced a decrease of FOXM1, p-FOXM1, and CCNB1 expression at 24 and 48 hours in a time-dependent manner. FOXA1 expression decreased markedly at 24 and 48 hours, while FOXO3A expression increased at 24 hours and then decreased at 48 hours.

NB compounds promote FOXM1 proteasomal degradation in HGSC cells

To determine the mechanism mediating reduced expression of FOXM1 by NB compounds, we co-treated OVCAR4 or COV318 cells with the NB compounds and the proteasome inhibitor MG132. We found that MG132 was toxic in CAOV3 cells, so COV318 cells served as a more suitable HGSC model for this experiment. In both OVCAR4 (**Figure 3A**) and COV318 (**Figure 3B**) cells, FOXM1 expression markedly decreased upon treatment with 2.5 μ M NB-73 or NB-115 but was rescued when proteasomal degradation was inhibited. This observation is consistent with our previously reported observations in breast cancer cells, where FOXM1 protein in breast cancer cell extracts treated with NB-73 was more susceptible to degradation by pronase, suggesting the NB compounds destabilize FOXM1 protein, and MG132 reversed the downregulation of FOXM1 by NB-73 in MDA-MB-231 cells²⁸.

It has been previously reported that proteasome inhibition decreases FOXM1 expression¹⁶. Indeed, we did observe this phenomenon in some of the HGSC cell lines we tested. In fact, it appeared that this effect was dependent on both the cell line and MG132 concentration (**Supplementary Figure S1**). We also found that treatment with 1 μ M MG132 for 24 hours was toxic in many, but not all, HGSC cell lines. It has been previously reported that the upregulation of HSP70, which is caused by the inhibition of its degradation, is responsible for the degradation of FOXM1 by proteasome inhibitors¹⁷. HSP70 purportedly binds to the FOXM1 DNA binding domain, inhibiting the ability of FOXM1 to promote its own transcription¹⁷. However, our previous studies were not able to confirm this positive autoregulatory loop in several cell types, including HGSC cells¹⁰. While we also observed an increase in HSP70 expression with proteasome inhibition, this did not consistently correlate with a decrease in FOXM1 expression (**Supplementary Figure S1**). From our data, we cannot conclude whether the decrease in FOXM1 expression by proteasome inhibition is caused by the upregulation of HSP70, but our results suggest that the concentration of MG132 is critical when studying this phenomenon and FOXM1 expression generally.

Interestingly, after six hours treatment, NB-55 caused a decrease in p-FOXM1 expression with minimal change in FOXM1 expression in both CAOV3 and OVCAR4 cells (**Figure 1A-B**), suggesting that NB-55 may promote a decrease in phosphorylated FOXM1 prior to FOXM1 degradation. We further explored the potential ability for NB compounds to modulate FOXM1 phosphorylation by examining their effects on p-FOXM1 and FOXM1 expression one hour after treatment (**Supplementary Figure S2**). In CAOV3 cells, the ratio of p-FOXM1 to FOXM1 expression remained ~ 1 regardless of treatment. In OVCAR4 cells, p-FOXM1 expression does not change while FOXM1 expression varies marginally, potentially as a result of slight loading differences. Thus, the NB compounds appear to primarily function by reducing overall FOXM1 protein levels.

NB compounds promote HGSC cellular apoptosis

To investigate the ability for the NB compounds to promote HGSC cell death, we examined the apoptotic activity of CAOV3, OVCAR4, and FT282 C11 cells treated with NB-73 and NB-115 for 24 hours. In CAOV3 cells (**Figure 4A**), NB-73 demonstrated a dose-dependent increase in caspase 3/7 activity, a common measure of cellular apoptosis. NB-115 did not show this dose-dependent increase, possibly because maximum effect was already reached at 1 μ M NB-115. In OVCAR4 cells (**Figure 4B**), a dose-dependent increase in caspase 3/7 activity was observed for both NB-73 and NB-115. While HGSC cells treated with NB-73 and NB-115 demonstrated increased caspase 3/7 activity, FT282 C11 did not (**Figure 4C**), further demonstrating the selectivity of NB-73 and NB-115 for HGSC cells. The pan-caspase inhibitor Q-VD-OPh decreased caspase 3/7 activity in CAOV3 cells (**Figure 4D**) and rescued CAOV3 cells from cell death (**Figure 4E**). Visually, CAOV3 cells showed remarkable cell death when treated with NB-73 and NB-115 (**Figure 4G,I**) compared to DMSO control treatment (**Figure 4F**). The visual cell death induced by NB-73 and NB-115 was prevented when cells were co-treated with Q-VD-OPh (**Figure 4H,J**). Representative apoptosis profiles generated by flow cytometry for can be found in **Supplemental Figure 3**.

NB compounds inhibit HGSC colony formation

To examine the ability of the NB compounds to inhibit two-dimensional, anchorage-dependent HGSC colony formation (i.e., clonogenic growth), CAOV3, OVCAR4, and OVCAR5 cells were treated with the most potent NB compounds, NB-73 and NB-115, and allowed to form colonies. NB-73 decreased colony formation at concentrations between 50 and 200 nM in CAOV3, OVCAR4, and OVCAR5 cells, with 200 nM NB-73 yielding virtually no colonies (**Figure 5A**). NB-115 decreased colony formation at concentrations between 25 and 200 nM in CAOV3 cells and between 12.5 and 200 nM in OVCAR4 cells, with 200 nM NB-115 yielding virtually no colonies in both cell lines (**Figure 5B**). OVCAR5 cells needed higher concentrations of NB-115 to yield the same suppressive effect on colony formation as in CAOV3 and OVCAR4 cells. In OVCAR5 cells, concentrations between 50 and 200 nM NB-115 colony formation; however, >200 nM NB-115 would be necessary to fully suppress colony formation in OVCAR5.

For comparison, we also examined the ability of thiostrepton and FDI-6 to inhibit two-dimensional, anchorage-dependent colony formation in CAOV3 and OVCAR4 cells. Thiostrepton decreased colony formation at concentrations between 25 and 200 nM in CAOV3 cells, with ~10% colonies compared to control at 200 nM thiostrepton, and between 0.25 and 2 μ M in OVCAR4 cells (**Figure 5C**), with 2 μ M yielding virtually no colonies. Unexpectedly, FDI-6 did not suppress colony formation in either CAOV3 or OVCAR4 cells, even at 100 μ M concentrations (**Figure 5D**).

To examine the ability of the NB compounds to inhibit three-dimensional, anchorage-independent HGSC colony formation, OVCAR5 and OVCAR8 cells in soft agar were treated with NB-73 or NB-115 and allowed to form colonies. CAOV3 and OVCAR4 cells were not able to form colonies in soft agar and were, therefore, not used in the analysis. NB-73 (**Figure 5E**) decreased colony formation at concentrations between 100 and 400 nM in OVCAR5 cells, with 400 nM NB-73 suppressing all colony formation, and between 200 and 600 nM in OVCAR8 cells, with 600 nM NB-73 suppressing all colony formation. Similarly, NB-115 (**Figure 5F**) decreased colony formation at concentrations between 200 and 500 nM in OVCAR5 cells, with 500 nM NB-115 suppressing all colony formation, and between 200 and 600 nM in OVCAR8 cells, with 600 nM NB-115 suppressing all colony formation.

NB-73 and NB-115 synergize with carboplatin to impair HGSC cell viability

To investigate the potential use of the NB compounds in combination with relevant HGSC chemotherapies, we investigated the synergy between NB-73 or NB-115 with the platinum-based chemotherapy agent carboplatin or the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib. Combinations with these chemotherapy agents are supported by the known role of FOXM1 in stimulating the expression of DNA repair genes⁸ as well as our recent studies using genetic depletions of FOXM1⁴⁴. The synergy results are detailed in **Table 1**. The dose-effect curves can be found in **Supplementary Figure S5** and logarithmic combination index plots can be found in **Supplementary Figure S6**. The combination index (CI) of the effective doses (ED) for 75%, 90% and 95% of the population was averaged to create a CI_{average} . Synergistic effects are demonstrated between the two drugs when $CI < 1$, additive effects are demonstrated when $CI = 1$, and antagonistic effects are demonstrated when $CI > 1$. Carboplatin with NB-73 showed synergy in both CAOV3 cells ($CI_{\text{average}} = 0.72$) and OVCAR4 cells ($CI_{\text{average}} = 0.84$). Carboplatin

with NB-115 showed antagonism in CAOV3 cells ($CI_{\text{average}} = 1.68$) and synergy in OVCAR4 cells ($CI_{\text{average}} = 0.65$). Conversely, olaparib with NB-73 showed antagonism in both CAOV3 cells ($CI_{\text{average}} = 3.90$) and OVCAR4 cells ($CI_{\text{average}} = 1.40$). Similarly, olaparib with NB-115 showed antagonism in CAOV3 cells ($CI_{\text{average}} = 2.23$) and additive effects in OVCAR4 cells ($CI_{\text{average}} = 1.00$).

NB-73 and NB-115 show superior selectivity and cytotoxicity in HGSC cells among a panel of tested FOXM1 inhibitors

To determine the potency and efficacy of the NB compounds compared to other reported FOXM1 inhibitors, we examined the effect of a panel of FOXM1 inhibitors on cell viability of CAOV3 and OVCAR4 cells. Potency was defined by the IC_{50} value, and efficacy was defined by the loss of cell viability compared to control cells treated with solvent only. The IC_{50} value for NB-55 (**Figure 6B**) was in the low micromolar range compared to the mid-nanomolar IC_{50} values for NB-73 (**Figure 6C**) and NB-115 (**Figure 6D**). FDI-6 (**Figure 6E**) demonstrated a low micromolar IC_{50} value and somewhat low efficacy, and thiostrepton (**Figure 6F**) had a high nanomolar IC_{50} value. Monensin had a low nanomolar IC_{50} value but was not particularly efficacious in OVCAR4 cells and demonstrated a broad dose-response curve in CAOV3 cells (**Figure 6G**). N-phenylphenanthren-9-amine (**Figure 6H**) had a low micromolar IC_{50} value, though at least 2-fold higher than that observed for FDI-6. RCM-1 (**Figure 6I**) had no effect on HGSC cell viability, consistent with observations in breast cancer cell lines (data not shown). The IC_{50} values and efficacy rates are summarized in **Table 2**, which is stratified by the most potent and efficacious compounds (NB-73 and NB-115) to the least potent and efficacious compounds (N-phenylphenanthren-9-amine and RCM-1).

To explore the selectivity of the NB compounds for FOXM1 and for HGSC cells versus non-malignant cells, we used the immortalized fallopian tube epithelium (FTE) cell line FT282 C11 to serve as a normal cell control highly relevant to HGSC^{31,32}. Notably, in characterizing FT282 C11 cells for FOXM1, p-FOXM1, CCNB1, FOXA1, and FOXO3A expression, FT282 C11 cells had markedly lower FOXM1, p-FOXM1, CCNB1, and FOXA1 protein expression and markedly higher FOXO3A protein expression compared to CAOV3 and OVCAR4 cells (**Figure 6A**). NB-55 (**Figure 6B**) showed no selectivity for HGSC over FT282 C11 cells. In contrast, NB-73 (**Figure 6C**) and NB-115 (**Figure 6D**) showed an average of 10-fold and 7-fold selectivity, respectively, for HGSC cells. Due to their widespread use in the FOXM1 field, we also investigated the effects of thiostrepton and FDI-6. FDI-6 (**Figure 6E**) showed a moderate selectivity for HGSC cells at an average of 1.5-fold, and thiostrepton (**Figure 6F**) showed no selectivity between HGSC and FT282 C11 cells. The IC_{50} values and efficacy rates are summarized in **Table 3**, which is stratified by most selective compound (NB-73) to least selective compound (NB-55 and thiostrepton) for HGSC cells.

Discussion

In this study, we demonstrated that the NB compounds promote several anti-cancer effects in HGSC cells that reflect favorably on their therapeutic potential for the treatment of HGSC cancers. The NB

compounds promoted FOXM1 proteasomal degradation in HGSC cells, an effect that appears to be selective for FOXM1 as other FOX proteins were not similarly affected, and they potently and efficaciously decreased HGSC cell viability, in large part by promoting apoptosis. The NB compounds have shown similar activities in TNBC cells²⁸, and consistent with observations in TNBC cells²⁸, the 1,1-diarylethylene diamine methiodide salts NB-73 and NB-115 were more potent FOXM1 inhibitors and suppressors of HGSC cell viability than the 1,1-diarylethylene monoamine methiodide NB-55. The NB compounds also suppressed two-dimensional, anchorage-dependent and three-dimensional, anchorage-independent HGSC colony formation at nanomolar concentrations. As the colony formation assay requires colonies to form from a single cell, these results suggest the NB compounds may exert anti-cancer effects on tumor cells growing at metastatic sites in addition to cancer cells in the primary tumor.

Ample evidence indicates that FOXM1 promotes chemotherapy resistance, including platinum-based chemotherapy resistance, in ovarian cancer⁸. Here, we found that the NB compounds synergized with the platinum-based chemotherapy agent carboplatin, a mainstay treatment for ovarian cancer and several other cancers, to inhibit HGSC cell viability, offering the potential for combination therapy in the clinic. In breast cancer cells that have become resistant to NB compound treatment, transcriptional processes had become rewired such that breast cancer cells became sensitive to lapatinib, an anti-EGFR therapy used in breast cancer⁴⁵. Therefore, cancer treatment with the NB compounds shows promise in being used in combination as well as consecutively with existing chemotherapy.

Curiously, FOXA1 and FOXO3A expression appeared to show some changes in response to treatment with FOXM1 inhibitors. However, these expression changes were generally modest and transient, and the observations were not consistent between cell lines. The tumor suppressor FOXO3A is well-known to downregulate FOXM1 expression through several mechanisms⁷, but FOXM1 affecting FOXO3A expression has not been reported. Similarly, the relationship between FOXM1 and FOXA1 expressions has not been studied.

Thiostrepton required 48 hours in CAOV3 cells to downregulate CCNB1 expression and did not differentiate between FOXM1-high HGSC cells and FOXM1-low immortalized FTE cells in reducing cell viability. While thiostrepton was able to suppress colony formation in HGSC cells, the effective concentrations exhibited a broad range and were highly dependent on the HGSC cell line, despite FOXM1 overexpression. As a thiazole antibiotic, thiostrepton is known to have pleiotropic effects, such as disruption of mitochondrial protein synthesis⁴⁶. Therefore, although thiostrepton did downregulate FOXM1 expression and, consequently, CCNB1 expression, our results suggest that the ability of thiostrepton to downregulate FOXM1 expression may not be the primary mechanism through which it exerts its biological effects.

In contrast to the NB compounds decreasing intracellular FOXM1 protein levels, we observed that FDI-6 did not substantially reduce FOXM1 protein expression in HGSC cells and only modestly inhibited the downstream CCNB1 expression. This observation is consistent with the reported inhibitory effects of FDI-6 on FOXM1 function without inducing FOXM1 protein degradation¹⁸. Consequently, FDI-6 may not be

very effective at inhibiting cancer phenotypes. Indeed, FDI-6 demonstrated limited efficacy (~60%) when treating HGSC cells for 72 hours and did not affect HGSC colony formation over the course of two weeks. However, FDI-6 was recently used as a lead compound to create a proteolysis-targeting chimera (PROTAC), which promotes the degradation of FOXM1 protein⁴⁷, an approach that may provide a better mechanism for long-term, therapeutic effect. Furthermore, FDI-6 was not particularly potent, affecting HGSC cell viability in the low micromolar range. A more potent molecule based on FDI-6 was recently generated⁴⁸, which may offer more opportunity for further study of this class of FOXM1 inhibitor.

The other FOXM1 inhibitors we explored were either not efficacious (monensin and RCM-1) or considerably less potent (N-phenylphenanthren-9-amine) than the NB compounds. The disparity between the dose-response curve produced in CAOV3 and OVCAR4 cells treated with monensin suggests that pleiotropic effects are exerted to produce the biological response on cell viability. This observation is not surprising, as monensin is well-known to block Golgi transport and protein secretion^{49,50} and is extensively used for this purpose in laboratory research. As an ionophore that selectively transports sodium cation across lipid membranes, monensin has also been shown to induce mitochondrial ROS production and disrupt calcium homeostasis⁵¹. Surprisingly, RCM-1 had no effects on HGSC (or breast cancer) cell viability. However, it was reported that RCM-1 did affect cell viability in several other cancer cell lines⁵². RCM-1, which has structural similarity to FDI-6, was initially shown to translocate nuclear FOXM1 into the cytoplasm of U2OS cells, resulting in the proteasomal degradation of FOXM1²⁷. Our results suggest that further validation as to how RCM-1 affects cancer cell viability is warranted. Aside from RCM-1, which had no effect on HGSC cell viability, N-phenylphenanthren-9-amine was the least potent compound out of all the FOXM1 inhibitors we tested.

We were limited in our comparison of the NB compounds to the most widely used and accessible reported FOXM1 inhibitors. Although many natural compounds have been shown to inhibit FOXM1⁵³⁻⁵⁵, their anti-cancer effects may not be due to inhibition of FOXM1 given their likely pleiotropic nature. Other FOXM1 inhibitors, such as 7-difluoromethoxyl-5,4'-di-n-octyl-genistein (which was synthesized from the natural compound genistein)⁵⁶, are not currently commercially available. Additionally, several new FOXM1 inhibitors have been recently reported^{47,48,57,58}, demonstrating the high interest in therapeutically targeting FOXM1 for cancer treatment. Our study establishes the NB compounds as novel FOXM1 inhibitors that merit further investigation for their clinical translatability to treat aggressive cancers that overexpress FOXM1, such as HGSC.

Conclusion

The 1,1-diarylethylene diamine methiodide salt FOXM1 inhibitors NB-73 and NB-115 promoted proteasomal degradation of FOXM1, reduced HGSC survival by promoting apoptosis, and selectively inhibited HGSC cell viability as compared to normal control fallopian tube epithelial cells. NB-73 and NB-115 potently suppressed both two-dimensional, anchorage-dependent and three-dimensional, anchorage-independent HGSC colony formation. These NB compounds also demonstrated synergy with the

platinum-based chemotherapy agent carboplatin, frequently used in treatment of HGSC. In a panel of previously characterized FOXM1 inhibitors, NB-73 and NB-115 emerged as the most potent and selective suppressors of HGSC cell viability. Our findings characterize the activities of the NB compounds as potent and selective FOXM1 inhibitors and should encourage assessment of their therapeutic potential for the treatment of the vast majority of HGSC and other aggressive cancers that overexpress FOXM1.

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Declarations

Acknowledgements

We thank Dr. Amarnath Natarajan for helpful advice, Ms. Smitha Kizhake for assistance with drug synergy experiments, and Dr. Jennifer Black for critical reading of the manuscript.

Funding

NIH T32CA009476 (C.L.), research grants 083 (B.S.K.) and 084 (J.A.K.) from the Breast Cancer Research Foundation, the Landfield Cancer Research Fund (B.S.K.), NIH R03CA224339 (A.R.K.), the Marsha Rivkin Center (A.R.K.), the Betty J. and Charles D. McKinsey Ovarian Cancer Research Fund (A.R.K.), and the Fred & Pamela Buffett Cancer Center (NCI P30CA036727) (A.R.K.).

Author contributions

C.L. and A.R.K. conceived and designed the study. C.L. and C.M.-T. performed the experiments and collected and analyzed the experimental data. C.L. and A.R.K. interpreted the experimental results with additional support from J.A.K. and B.S.K. S.H.K., J.A.K., and B.S.K. generated the NB compounds. C.L. drafted the manuscript and prepared all figures. All authors reviewed, revised, and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics declarations

Competing Interests

S.H.K., J.A.K., and B.S.K. are coinventors on the patent application filed by the University of Illinois to cover the NB-55, NB-73, and NB-115 compounds described in this paper. The other authors declare no conflicts of interest.

Additional information

None.

Tables

Table 1. Synergy of NB-73 and NB-115 with existing ovarian cancer chemotherapy agents in CAOV3 and OVCAR4 cells.

Drug Combination	HGSC Cell Line	ED ₇₅	ED ₉₀	ED ₉₅	CI Average
Carboplatin + NB-73	CAOV3	1.11	0.62	0.42	0.72
	OVCAR4	1.23	0.75	0.54	0.84
Carboplatin + NB-115	CAOV3	2.25	1.57	1.23	1.68
	OVCAR4	0.94	0.59	0.43	0.65
Olaparib + NB-73	CAOV3	5.34	3.60	2.75	3.90
	OVCAR4	1.26	1.41	1.52	1.40
Olaparib + NB-115	CAOV3	2.15	2.24	2.31	2.23
	OVCAR4	1.36	0.92	0.71	1.00

Table 2. Potency and efficacy of various FOXM1 inhibitors on CAOV3 and OVCAR4 cell viability.

Compound Name	CAOV3		OVCAR4	
	IC ₅₀ (μM) ± SE	% Efficacy*	IC ₅₀ (μM) ± SE	% Efficacy*
NB-73	0.60 ± 0.17	98%	0.34 ± 0.06	86%
NB-115	0.49 ± 0.19	100%	0.56 ± 0.09	85%
Thiostrepton	0.62 ± 0.03	93%	0.98 ± 0.14	99%
NB-55	3.0 ± 0.09	89%	3.2 ± 0.15	96%
FDI-6**	9.6 ± 0.73	61%	4.7 ± 0.26	61%
Monensin	0.10 ± 0.15	100%	0.024 ± 0.01	52%
N-phenylphenanthren-9-amine	23 ± 1.9	85%	39 ± 5.2	100%
RCM-1	N/A	0%	N/A	0%

*Efficacy is defined as loss of cell viability shown by the CyQUANT™ proliferation assay.

**FDI-6 is a fluorescent molecule that interferes with the CyQUANT™ proliferation assay readout. IC₅₀ values are likely slightly overestimated and % efficacy values are likely slightly underestimated.

Table 3. Potency and efficacy of various FOXM1 inhibitors on CAOV3 and OVCAR4 compared to FT282 cell viability.

Compound Name	CAOV3		OVCAR4		FT282 C11		Tumor-to-Normal Ratio***
	IC ₅₀ (μM)	% Efficacy*	IC ₅₀ (μM)	% Efficacy*	IC ₅₀ (μM)	% Efficacy*	
NB-73	0.60	98%	0.34	86%	6.0	81%	0.078
NB-115	0.49	100%	0.56	85%	3.8	100%	0.14
FDI-6**	9.1	61%	4.7	61%	11	76%	0.63
Thiostrepton	0.62	93%	0.98	99%	0.73	100%	1.1
NB-55	3.0	89%	3.2	96%	2.8	96%	1.1

*Efficacy is defined as loss of cell viability shown by the CyQUANT™ proliferation assay.

**FDI-6 is a fluorescent molecule that interferes with the CyQUANT™ proliferation assay readout. IC₅₀ values are likely slightly overestimated and % efficacy values are likely slightly underestimated.

***Tumor-to-normal ratio was calculated by averaging the IC₅₀ values of CAOV3 and OVCAR4 cells and then dividing by the IC₅₀ value of FT282 C11.

Figures

Figure 1

Effect of NB-55 and NB-73 on the protein expression of FOXM1, the FOXM1 signaling pathway, and other FOX family members in HGSC cells.

Western blot analysis of FOXM1, p-FOXM1 (threonine 600 phosphorylation site; activated FOXM1), CCNB1 (downstream target of FOXM1), FOXA1 (oncogenic FOX family member), and FOXO3A (tumor suppressor FOX family member) expression are shown. (A) CAOV3 cells were treated with 6, 7, and 8 μ M NB-55 for 6 hours, 24 hours, and 48 hours. (B) OVCAR4 cells were treated with 7 and 8 μ M NB-55 for 6 hours, 24 hours, and 48 hours. (C) CAOV3 were treated with 1 and 2.5 μ M NB-73 for 6 hours, 24 hours, and 48 hours. (D) OVCAR4 cells were treated with 1 and 2.5 μ M NB-73 for 24 hours and 48 hours. Protein quantification is provided below each protein band, and protein expression has been normalized to the control treatment at the appropriate time point. Full image, original blots can be found in **Supplementary Figure S7**. Ponceau S stains show protein loading. Protein levels were quantified using Fiji software³³.

Figure 2

Effect of NB-115, FDI-6, and thiothrepton on the protein expression of FOXM1, the FOXM1 signaling pathway, and other FOX family members in HGSC cells.

Western blot analysis of FOXM1, p-FOXM1 (threonine 600 phosphorylation site; activated FOXM1), CCNB1 (downstream target of FOXM1), FOXA1 (oncogenic FOX family member), and FOXO3A (tumor suppressor FOX family member) expression are shown. **(A)** CAOV3 were treated with 1 and 2.5 μ M NB-115 for 6 hours, 24 hours, and 48 hours. **(B)** OVCAR4 cells were treated with 1 and 2.5 μ M NB-115 for 24 hours and 48 hours. **(C)** CAOV3 and **(D)** OVCAR4 cells were treated with 2.5 μ M thiothrepton (Thio) or 20 μ M FDI-6 for 24 hours and 48 hours. Protein quantification is provided below each protein band, and protein expression has been normalized to the control treatment at the appropriate time point. Full image, original blots can be found in **Supplementary Figure S7**. Ponceau S stains show protein loading. Protein levels were quantified using Fiji software³³.

Figure 3

Proteasome inhibition reverses effects of NB compounds on FOXM1 in HGSC cells.

Western blot analysis of FOXM1 and ubiquitin expression in (A) OVCAR4 and (B) COV318 cells treated for 24 hours with DMSO, NB-73, NB-115, MG132 (proteasome inhibitor), NB-73 and MG132, and NB-115 and MG132. Protein quantification is provided below each protein band, and protein expression has been normalized to the DMSO control treatment. Full image, original blots can be found in **Supplementary Figure S8**. Ponceau S stains show protein loading. Protein levels were quantified using Fiji software³³ and graphed using GraphPad Prism 7 software.

Figure 4

NB-73 and NB-115 promote apoptosis in CAOV3 and OVCAR4 cells and can be reversed with pan-caspase inhibitor Q-VD-OPh.

Apoptosis of (A) CAOV3, (B) OVCAR4, and (C) FT282 C11 cells after 24 hours of treatment with DMSO, 1 μM NB-73, 2.5 μM NB-73, 1 μM NB-115, and 2.5 μM NB-115 are graphed. (D) Apoptotic and (E) live cells of CAOV3 cells after 48 hours of treatment with DMSO, 1 μM NB-73, 1 μM NB-73 and 20 μM Q-VD-OPh (pan-caspase inhibitor), 1 μM NB-115, and 1 μM NB-115 and 20 μM Q-VD-OPh are graphed. Apoptotic and live cells were evaluated using the Muse® Caspase-3/7 Kit followed by flow cytometry using the Guava® Muse® Cell Analyzer. Assays were run in three independent trials. Values are mean ± SEM. In A, B, D, and E, cellular responses to NB-73 treatment are represented in shades of red, while cellular responses to NB-115 treatment are represented in shades of blue. In C, caspase 3/7 activity of FT282 C11 cells (tan) is shown in comparison to CAOV3 cells (red) and OVCAR4 cells (blue), and *p*-values denote comparison to DMSO control only. In GraphPad Prism 7 software, *p*-values were calculated using one-way ANOVA with Sidak's multiple comparisons test (A,B) or Tukey's multiple comparison's test (D,E) or using two-way ANOVA with Dunnett's multiple comparison's test (C). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Representative images of CAOV3 cells treated for 48 hours with (F) DMSO, (G) 1 μM NB-73, (H) 1 μM NB-73 and 20 μM Q-VD-OPh, (I) 1 μM NB-115, and (J) 1 μM NB-115 and 20 μM Q-VD-OPh are shown at 10X magnification. The red bar represents 600 μm.

Figure 5

Effect of NB compounds, thioestrepton, and FDI-6 on two-dimensional, anchorage-dependent and three-dimensional, anchorage-independent HGSC colony formation.

For two-dimensional, anchorage-dependent colony formation, CAOV3, OVCAR4, and OVCAR5 cells were treated with NB-73, NB-115, thioestrepton, or FDI-6 in triplicate wells and allowed to form colonies for 7-14 days, depending on the cell line. For three-dimensional, anchorage-independent colony formation, OVCAR5 and OVCAR8 cells were treated with NB-73 or NB-115 and allowed to form colonies in soft agar for 14 days. Following incubation, cells were stained with crystal violet. Colonies were quantified using countPHICS (count and Plot Histograms of Colony Size) software³⁴, where the threshold for colony size was automatically determined. Graphs were generated using GraphPad Prism 7 software. For two-dimensional, anchorage-dependent colony formation, graphs are shown of CAOV3 (red), OVCAR4 (blue), and OVCAR5 (purple) cells in response to treatment with (A) NB-73, (B) NB-115, (C) thioestrepton, or (D) FDI-6. For three-dimensional, anchorage-independent (soft agar) colony formation, graphs are shown of OVCAR5 (purple) and OVCAR8 (orange) cells in response to treatment with (E) NB-73 or (F) NB-115. Values are mean $\pm$ SEM. Representative images of colony formation for each concentration can be found in **Supplementary Figure 4**.

Figure 6

Expression of FOXM1, the FOXM1 signaling pathway, and other FOX family members in HGSC and FT282 C11 cells and dose-response curves of HGSC and FT282 C11 cells treated with various FOXM1 inhibitors.

(A) Western blot analysis of FOXM1, p-FOXM1 (threonine 600 phosphorylation site; activated FOXM1), CCNB1 (downstream target of FOXM1), FOXA1 (oncogenic FOX family member), and FOXO3A (tumor suppressor FOX family member) expression in CAOV3 (HGSC cell line), OVCAR4 (HGSC cell line), and FT282 C11 (immortalized fallopian tube epithelium cell line; normal cell control highly relevant to HGSC). Protein quantification is provided below each protein band, and protein expression has been normalized to the average of CAOV3 and OVCAR4 protein levels. Full image, original blots can be found in **Supplementary Figure 9**. Ponceau S stain show protein loading. Protein levels were quantified using Fiji software³³. Dose-response curves of CAOV3 (red) and OVCAR4 (blue) cells treated for 72 hours with (B) NB-55, (C) NB-73, (D) NB-115, (E) FDI-6, (F) thiostrepton, (G) monensin, (H) N-phenylphenanthren-9-amine, and (I) RCM-1. Cell viability was analyzed using CyQUANT™ Cell Proliferation Assay, for cells in culture. Three independent trials were performed with three technical replicates per trial. Values represent mean ± SEM. Curves were created, when applicable, using nonlinear regression with least squares (ordinary) fit in GraphPad Prism 7 software.

Supplementary Files

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