Targeting Prostate Cancer Subtype 1 by Forkhead Box M1 Pathway Inhibition

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Abstract

Purpose: Prostate cancer was recently classified to three clinically relevant subtypes (PCS) demarcated by unique pathway activation and clinical aggressiveness. In this preclinical study, we investigated molecular targets and therapeutics for PCS1, the most aggressive and lethal subtype, with no treatment options available in the clinic.

Experimental Design: We utilized the PCS1 gene set and our model of enzalutamide (ENZ) castration-resistant prostate cancer (CRPC) to identify targetable pathways and inhibitors for PCS1. The findings were evaluated in vitro and in the ENZCRPC xenograft model in vivo.

Results: The results revealed that ENZCRPC cells are enriched with PCS1 signature and that Forkhead box M1 (FOXM1) pathway is the central driver of this subtype. Notably, we identified Monensin as a novel FOXM1-binding agent that selectively targets FOXM1 to reverse the PCS1 signature and its associated stem-like features and reduces the growth of ENZ CRPC cells and xenograft tumors.

Conclusions: Our preclinical data indicate FOXM1 pathway as a master regulator of PCS1 tumors, namely in ENZ CRPC, and targeting FOXM1 reduces cell growth and stemness in ENZ CRPC in vitro and in vivo. These preclinical results may guide clinical evaluation of targeting FOXM1 to eradicate highly aggressive and lethal PCS1 prostate cancer tumors. Clin Cancer Res; 23(22): 11–11. © 2017 AACR.

Introduction

Prostate cancer is the second leading cause of cancer death among Western men (1). Androgen deprivation therapy targeting androgen receptor (AR) and blocking its signaling is the cornerstone of therapies for prostate cancer patients with metastatic and castration-resistant disease (2, 3). These include second-line therapies, namely enzalutamide (ENZ) and abiraterone acetate, that improve survival of patients with castration-resistant prostate cancer (CRPC; refs. 4, 5). Nevertheless, the effects are not curative, and resistance to these therapies rapidly occurs (6, 7). Recent advances in genotyping CRPC have underlined the role of heterogeneity in reactivation of AR activity: the AR-driven resistance in CRPC remains dependent on AR signaling: for example, via emergence of AR point mutations and splice variants (such as AR-V7) leading to acquired resistance to androgen deprivation therapies (3, 6, 8–12). Moreover, cross-talk with other signaling pathways that drive AR activity has been described (6, 8). In contrast, "AR-Indifferent" disease, where the resistant cells lack AR expression and/or signaling activity, has recently been reported to be associated with cellular plasticity and neuroendocrine molecular features (13).

Due to acquired resistance and the significant biological heterogeneity seen in prostate cancer tumors, there has long been a clinical need to identify master regulators that could be targeted to treat the most lethal, aggressive prostate cancer. In a recent study, prostate cancer was classified to three prostate cancer subtypes (PCS), PCS1, PCS2, and PCS3, by utilizing and integrating multiple publically available prostate cancer gene expression data sets (n > 4,600; ref. 14). The luminal-like type 1 signature PCS1 was characterized as the most aggressive and lethal form of prostate cancer (14). Interestingly, analysis of the circulating tumor cells from ENZ-resistant (ENZ⁸) patient’s revealed that most of the ENZ⁸ patients belonged to the PCS1 subtype (14, 15). However, there are no molecular targets or therapeutic options for patients with PCS1 tumors in the clinic.

In this study, we sought to identify novel targets and therapeutics for the most lethal prostate cancer subtype, PCS1. To achieve this goal, we utilized the PCS classifiers and our recently generated models of ENZ⁸ CRPC (14, 16) and found FOXM1 as a therapeutic target for tumors with a PCS1 subtype.

Materials and Methods

Cells

The prostate carcinoma CRPC and ENZ⁸ CRPC cells were generated from LNCaP as previously reported (16, 17), tested and authenticated by whole-genome and whole-transcriptome sequencing (Illumina Genome Analyzer IIX, 2012), and tested as free of mycoplasma contamination and grown in RPMI 1640/10% FBS/1% glutamate/1% penicillin–streptomycin (Hyclone), and 10 μmol/L ENZ or DMSO.

Compounds

Enzalutamide (ENZ; Haoyuan Chemexpress) and Thiostrepton (Sigma-Aldrich) were diluted in DMSO (Sigma-Aldrich), and

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Monensin (Mon; Sigma-Aldrich) was diluted in EtOH. ENZ concentration of 10 μmol/L, Thistrepton concentration of 100 nmol/L, and Mon concentration of 10 nmol/L were used in all experiments unless otherwise noted.

Cell proliferation assay

Cell proliferation assay was performed on 96-/384-well plates (Greiner) by platting 2,000/1,000 cells/well in 100/35 μL of media and left to attach overnight. Compound dilutions were added and incubated for 72 hours. Cell viability was determined with Cell-Titer-Glo (CTG, Promega, Inc.) and confluence with live-cell imaging (Incucyte, Essen Biosience Inc.). The luminescence signal (700 nm) from CTG was quantified with Teca 200 plate reader (Tecan).

Gene expression analysis using bead arrays

ENZ® CRPC cells were grown into approximately 70% confluence, and total RNA was extracted using TRIzol (Invitrogen). Integrity of the RNA was monitored prior to hybridization using a Bioanalyzer 2100 (Agilent) according to the manufacturer’s instructions. Note that 500 ng of purified RNA was amplified with the TotalPrep Kit (Ambion), and the biotin-labeled cDNA was hybridized to Agilent.

Analysis of gene expression data

Differentially expressed genes from microarray were set to a minimum fold change of > 1.5. The functional gene ontology, pathway annotations, and upstream regulator pathway analyses (z-score) were analyzed for the sets of differentially expressed genes using Ingenuity Pathway Analysis Software (Ingenuity Systems Inc.), and gene set enrichments were analyzed using MSigDB. In order to identify drugs with similar or opposite effects on gene expression, Connectivity Map and LINCS Canvas data-base, and Genesapiens database were utilized in FOXM1 pathway gene expressions’ analyses in prostate cancer patient samples and survival plots (18).

In silico transcriptomics analyses

Cancer Genome Browser, cBioPortal for Cancer Genomics database, and Genesapiens database were utilized in FOXM1 pathway gene expression analyses in prostate cancer patient samples and survival plots (19, 20).

Quantitative real-time PCR

Total RNA was extracted, and 2 μg of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Real-time monitoring of PCR amplification of cDNA was performed using DNA primers on ABI PRISM 7900 HT Sequence Detection System with SYBR PCR Master Mix (Applied Biosystems). GAPDH levels were used as an internal standard, and each assay was performed in triplicate.

Molecular docking

EADock DSS engine–based Swissdock web server was utilized to dock molecular structures of FOXM1 DNA–binding domain (pdb id: 3G73) and Mon (21). Mon chemical structure was searched from Zinc database, dockings were performed 5 times for each compound, and the results were analyzed using UCSF Chimera. Molecular dynamics (MD) simulations of Mon was performed starting from its docking poses in DNA-binding domain of the FOXM1 protein as predicted by Glide SP program (22). Mutant forms of FOXM1 (R236A and Y241A; K278A and H287A) were created using MOE (23). All MD simulations were performed with the CUDA-accelerated Amber 14 program. FOXM1 force field parameters were obtained from the ff14SB force field and the ligand; Mon parameters came from generalized amber force field with charges derived from an RESP fit using an HF/6-31G electrostatic potential calculated using the Gaussian 09 program. MD simulations were carried out within AMBER 14 on WestGrid facilities from Compute Canada (https://www.westgrid.ca). The production of MD simulation was conducted for 10 ns without any restraints under the NPT ensemble condition at a temperature of 298 K and pressure of 1 atm.

Mutagenesis of FOXM1

FOXM1 plasmid (DNASU) was double mutated on FOXM1-Mon–binding site (R236A_Y241A or K278A_H287A) using the Q5 Site-Directed Mutagenesis Kit (New England Biosciences) using following primers: R236A and Y241A: For: TACCTCGG-CATGGGCAATGATACACTC and Rev: GCCCGGGGCCTCA-GAGTTCGTCG; K278A and H287A: For: AACTCCATCTCGGCG-CAACCCCTGTCGAGC and Rev: CTTCACGGCTGGGGCG-GCAATGTCCTAAAGTAGG.

Chromatin immunoprecipitation

Cells treated with or without Mon (100 nmol/L) for 6 hours were cross-linked with PFA (Sigma-Aldrich) and sonicated to shear DNA. Chromatin immunoprecipitation (ChIp) assay was performed using the ChIP Assay Kit (Agrose Beads) according to the manufacturer’s protocol (Millipore) and antibody against FOXM1. The binding or FOXM1 to its target genes’ promoters, PLK1, CDC25B, AURKB, and CCNB1, was addressed using qPCR. The primers for promoters were PLK1 promoter, For: CCA-GAGGAGAAGATGTCCA and Rev: GTCCGTGTTCCTCGAAAAGC; CDC25B promoter, For: AAGACCGCTATCGTTCGGCGT and Rev: CCATTTTACAGACGGCAG; AURKB promoter, For: GGAGGAGGAAGATGTCG and Rev: CCCACGACGCACGGCAG; CCNB1 promoter, For: CGGGAATCATCCTCAAGCG and Rev: GGCGGCGCGACGCAAGAAG; and Actin control, For: AGCCGGCGCTACAGCTCA and Rev: CG-TAGGACACTCTCCATTAAGTGT.

Western blotting

Protein lysates (5–50 μg) were run on SDS-PAGE and transferred to nitrocellulose membranes, which were blocked in...
Data were acquired from 10,000 events on a Canto II (BD legend) for 60 minutes, or ALDH reagent according to the manufacturer's protocol. Cells were harvested. The same protocol as siRNA was used for transfection control. After 4 hours, OPTI-MEM media (Invitrogen) were replaced with complete media, and cells were incubated for 18 hours prior to second transfection. After 48 hours, FOXM1 indicator activity was measured using Dual-Luciferase Reporter Assay System (Promega) and microplate luminometer (Tecan) according to the manufacturer's instructions. All experiments were carried out in triplicate.

Cell transfections
42DENZR and 42FENZR cells were reverse transected on 96-well plates (20,000 cells/well) with FOXM1 promoter region cloned to pGL3-Basic Luciferase reporter plasmid (Promega) kindly provided by Dr. Pradip Raychaudhuri (University of Chicago; ref. 25) using Lipofectamine (0.5 µL/well; Invitrogen). Renilla luciferase reporter construct was used as a transfection control. After 24 hours, concentration series of Mon (10 to 100 nmol/L) and control dilutions were added onto the cells for 18 hours. FOXM1–luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) and microplate luminometer (Tecan) according to the manufacturer's instructions. All experiments were carried out in triplicate.

Flow cytometry
Cells were exposed to Mon for 24, 48, and 72 hours (PI) or for 24 hours [aldehyde dehydrogenase (ALDH) activity and CD24+/CD49b− staining], samples were stained with PI for 30 minutes at 4°C (subpopulation), CD24 and CD49b antibodies (1:20; Biolegend) for 60 minutes, or ALDH reagent according to the manufacturer's instructions (Stem Cell Technologies). Live cells were gated using staining with viability dye eFluor 506 (eBioscience). Data were acquired from 10,000 events on a Canto II (BD Biosciences). The results were analyzed using FlowJo (TreeStar).

Drug affinity responsive target stability assay
Cell lysates were incubated with vehicle or Mon, and the proteins were degraded with different concentrations of pronase according to the protocol as previously described (24). FOXM1 protein level was obtained using Western blot. GAPDH was used as a control.

Transfection and luciferase assay
42DENZR and 42FENZR cells were reverse transfected on 96-well plates (20,000 cells/well) with FOXM1 promoter region cloned to pGL3-Basic Luciferase reporter plasmid (Promega) kindly provided by Dr. Pradip Raychaudhuri (University of Chicago; ref. 25) using Lipofectamine (0.5 µL/well; Invitrogen). Renilla luciferase reporter construct was used as a transfection control. After 24 hours, concentration series of Mon (10 to 100 nmol/L) and control dilutions were added onto the cells for 18 hours. FOXM1–luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) and microplate luminometer (Tecan) according to the manufacturer's instructions. All experiments were carried out in triplicate.

Cell transfections
42DENZR and 42FENZR cells were plated on 10 cm plates (1 million cells/10 mL complete media; Corning Life Sciences) for 18 to 24 hours prior to transfection with 10 nmol/L FOXM1 or control siRNA (Santa Cruz Biotechnology) using Oligofectamine (Invitrogen) and OPTI-MEM media (Gibco). After 4 hours, OPTI-MEM media were replaced with complete media, and cells were incubated for 18 hours prior to second transfection. After 48 hours, cells were harvested. The same protocol as siRNA was used for shFOXMI transfections using shFOXMI or control shRNA (Santa Cruz Biotechnology), and successfully transfected clones were selected for and expanded in complete media containing 10 µg/mL puromycin. For transient FOXM1 overexpression, LNCaP and 16DCRPC cells were plated on 6-well plates, and FOXM1 plasmid (5 µg; DNASU) was transfected using Mirus T20/20 and OPTI-MEM media (Invitrogen) according to the manufacturer's instructions. OPTI-MEM media were replaced after 24 hours with complete media ± 10 µmol/L ENZ, and cells were harvested after 48 hours.

Flow cytometry
Cells were exposed to Mon for 24, 48, and 72 hours (PI) or for 24 hours [aldehyde dehydrogenase (ALDH) activity and CD24+/CD49b− staining], samples were stained with PI for 30 minutes at 4°C (subpopulation), CD24 and CD49b antibodies (1:20; Biolegend) for 60 minutes, or ALDH reagent according to the manufacturer's instructions (Stem Cell Technologies). Live cells were gated using staining with viability dye eFluor 506 (eBioscience). Data were acquired from 10,000 events on a Canto II (BD Biosciences). The results were analyzed using FlowJo (TreeStar).

Xenograft experiments
Athymic nude male mice (Harlan Sprague-Dawley), 5 weeks of age, were injected s.c. in the both flanks with 1 × 106 42DENZR cells in 200 µL of Matrigel without growth factors (BD Biosciences). Mice were castrated, and after 2 weeks of castration, mice were given ENZ (10 mg/kg/d). When tumor size reached 200 mm3, the mice were divided into two groups: (a) vehicle only and (b) Mon (10 mg/kg/3 times a week). Mice were treated for 3.5 weeks. Tumor volumes were calculated by caliper measurements twice a week to monitor tumor growth (tumor volume = LW2 × 0.56). For ALDH activity experiments, Mon (10 mg/kg/3 times a week) and ENZ (10 mg/kg/d) treatments started the next day after injections of the cells, and the tumors were harvested at the size of 100 to 300 mm3.

Statistical analyses
All in vitro and in vivo data were assessed using the Student t test. Disease-free survival was analyzed using Kaplan–Meier curves. Levels of statistical significance were set at *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Results
*AR-indifferent* ENZ® CRPC cells are enriched with PCS1 signature
Our gene expression profiling results showed that ENZ® CRPC 42DENZR and 42FENZR cells are enriched with PCS1 signature (gene expression fold changes were compared with 16DCRPC control cells, Fig. 1A and B), whereas PCS2 signature is significantly downregulated in both cells (Fig. 1A). The results were confirmed by gene set enrichment analyses (GSEA; Fig. 1C and Supplementary Fig. S1). Taken together, the analysis of 42DENZR and 42FENZR cells against the novel subtypes indicates that these cells are enriched with the most aggressive and lethal PCS, PCS1. Thus, these results reveal that our ENZ® cells could be further utilized to study novel targets and develop therapeutics for PCS1 patient subtype.

FOXM1 is a master regulator pathway in PCS1
To identify novel therapeutics for PCS1, we utilized the PCS1 gene set signature as well as the gene expression profiling of 42DENZR and 42FENZR cells and performed Connectivity Map (cMap) combined with LINCS analyses (18, 26) to identify compounds that could reverse all these three signatures (Fig. 2A). The results indicated that Thiostrepton, a Forkhead box M1 (FOXM1) inhibitor, is the most enriched compound reversing all three signatures (connectivity scores of −98, −92, and −90, in PCS1, 42DENZR, and 42FENZR, respectively). To confirm that the FOXM1 pathway is a master regulator in PCS1, we first confirmed that FOXM1 was upregulated in 42DENZR and 42FENZR cells compared with parental LNCaP and 16DCRPC cells (Supplementary Fig. S2A). Second GSEA analyses were performed, and the analysis revealed that the FOXM1 pathway is highly enriched and significantly activated in 42DENZR and 42FENZR (Fig. 2B; enrichment scores of 0.52 and 0.55 in 42DENZR and 42FENZR cells, respectively, P values < 0.01). Ingenuity upstream regulator pathway analysis also confirmed that the FOXM1 pathway was enriched in PCS1, 42DENZR, and 42FENZR cells with z-scores of 4.5, 3.8, and 3.3, respectively (Supplementary Fig. S2B). These data were further confirmed using qRT-PCR (Fig. 2C), showing that the FOXM1 pathway is upregulated in 42DENZR and 42FENZR.
When FOXM1 was overexpressed in LNCaP and 16DCRPC cells and represent 24% of PCS1 signature (Fig. 2D), and PCS1 was induced "AR-indifferent." Interestingly, the FOXM1 pathway was found to Supplementary Table S1), further suggesting that these cells are "AR-indifferent." In contrast, we found that AR (16), TP53, and RB1 pathways were downregulated in these cells with z-scores of −5.16, −5.02, and −4.15 for AR, TP53, and RB1, respectively (P values < 0.001; Supplementary Table S1), further suggesting that these cells are "AR-indifferent." Interestingly, the FOXM1 pathway was found to represent 24% of PCS1 signature (Fig. 2D), and PCS1 was induced when FOXM1 was overexpressed in LNCaP and 16DCRPC cells and downregulated when FOXM1 was silenced in 42DENZR and 42FENZR cells (Fig. 2E). Together, these data suggest that the FOXM1 pathway is a major regulator for PCS1.

Because PCS1 has been linked to high-risk prostate cancer, we next addressed whether the observed FOXM1 pathway activation in PCS1 subtype is also seen in high-risk prostate cancer patients compared with normal prostate samples (correlation coefficient of 0.51, P value < 0.001) as well as low PSA (correlation coefficient of −0.51, P value < 0.0001; Supplementary Table S2). In addition, high FOXM1 pathway expression was also seen in metastatic prostate cancer patients compared with primary patient samples analyzed using in Gresapiens database (Supplementary Fig. S2C; ref. 27). Finally, FOXM1, AURKB, PLK1, CCNB1, and SKP2 mRNA expressions correlate with poor survival in prostate cancer patients (Fig. 2G, analyzed from the data of ref. 28). In summary, these results from both unbiased compound and pathway analyses reveal FOXM1 as a major pathway activated in patients with PCS1 tumors as well as 42DENZR and 42FENZR cells enriched with PCS1. FOXM1 pathway activity correlated with high risk and poor survival, which is in accordance with the previous findings that PCS1 is the most aggressive and lethal subtype of prostate cancer and that FOXM1 activity is associated with poor survival, metastasis, and resistance to therapy (29, 30).

Mon is a novel FOXM1 and PCS1 subtype–targeting agent

As the Connectivity Map results revealed the FOXM1 inhibitor Thiostrepton as a potential agent targeting PCS1 tumors, we first explored its effect on 16DCRPC, 42DENZR, and 42FENZR cells. The results revealed that Thiostrepton reduces cell proliferation, and although differential effect was seen in 42DENZR and 42FENZR compared with 16DCRPC (P value < 0.01), the EC50 values were similar between the cell lines (Fig. 3A). The antiproliferative effect of Thiostrepton was observed at lower concentration compared with 40 μmol/L of its IC50 for FOXM1 indicating possible toxicity, a concern that has been reported previously (31, 32). Thiostrepton is a natural compound that belongs to a group of natural antibiotics produced by Streptomyces species. Notably, other natural antibiotics were also among the most enriched compounds for PCS1, 42DENZR, and 42FENZR including bithionol, Mon, manumycin, oligomycin, selamectin, idarubicin, and CCCP (Table 1). Thus, we tested the binding of these compounds to the FOXM1 DNA–binding domain (pdb id 3G73) using in silico Swissdock molecular docking (21). Thiostrepton–FOXM1-binding affinity was used as a reference (31). We found that Mon has


Figure 2.
FOXM1 is a master regulator pathway in PCS1. A, Common compounds reversing the gene expression signature of prostate cancer (PCa) patient PCS1 subtype and 42DENZR and 42FENZR cells analyzed using cMap and LINCS (18, 26). B, GSEA of the FOXM1 pathway in 42DENZR and 42FENZR cells. C, Expression of FOXM1, AURKB, PLK1, CCNB1, and SKP2 at mRNA levels by qRT-PCR in 16DCRPC, 42DENZR, and 42FENZR cells. *P < 0.05; **P < 0.01; and ***P < 0.001. D, The percentage of PCS1, PCS2, and PCS3 subtype-specific genes in the FOXM1 pathway. E, The effect of FOXM1 overexpression in LNCaP and 16DCRPC cells and silencing in 42DENZR and 42FENZR cells on PCS1 signature genes. F, The mRNA expression of FOXM1, AURKB, AURKA, BIRC5, PLK1, CCNB1, CCNA2, GTSE1, CCNE2, CDK1, CDK3, CENPA, and KLK3 in prostate cancer patients (TCGA, n = 550). G, Survival plots of FOXM1, AURKB, PLK1, SKP2, and CCNB1 in prostate cancer patient data analyzed using Taylor data set in cBioportal (28).

Targeting PCS1 by FOXM1 Pathway Inhibition

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Mon is a novel FOXM1 and PCS3 subtype-targeting agent. A, Relative cell proliferation of 16DCRPC, 42DENZR, and 42FENZR cells in response to various concentrations of Thiostrepton using CTG cell proliferation assay. Graph represents pooled data from three independent experiments. B, Effect of Mon (10 to 100 nmol/L) on FOXM1 transcriptional activity assessed by luciferase assay. C, Relative cell proliferation of 16DCRPC, 42DENZR, and 42FENZR cells in response to various concentrations of Mon. Graph represents pooled data from three independent experiments. D, Frequent contact maps of FOXM1-Mon binding reveal that Arg236, Tyr241, Lys278, and His287 interact with Mon over 80% of the total MD simulation time. E, Effect of Mon on mutated FOXM1 compared with WT as measured by transcriptional activity (FOXM1 activity P values of <0.01 and <0.001 for R236A_Y241A or K278A_H287A, respectively). F, Effect of Mon on mutated FOXM1 compared with WT as measured by cell proliferation (FOXM1 activity P values of <0.001 and <0.01 for R236A_Y241A or K278A_H287A, respectively). G, MD simulation showing that Mon could not form critical H-bond interactions with Tyr241 and His287 as they were mutated to Ala. H, Cells treated with or without Mon (100 nmol/L) for 6 hours were cross-linked with PFA (Sigma-Aldrich) and sonicated to shear DNA. ChIP assay was performed using the ChIP Assay Kit (Agarose Beads) according to the manufacturer’s protocol (Millipore) and antibody against FOXM1. The FOXM1 binding to its target genes’ promoters, PLK1, CDC25B, AURKB, and CCNB1 was evaluated using qPCR. I, Effect of Mon on the FOXM1 pathway in 42DENZR cells assessed by gene expression profiling and GSEA. J, mRNA expression of FOXM1 and its targets, AURKB, PLK1, SKP2, and CCNB1, assessed by qRT-PCR. Graph represents pooled data from three independent experiments. K, Effect of Mon on prostate cancer subtype signatures PCS1, PCS2, and PCS3 in 42DENZR cells analyzed by gene expression profiling and GSEA. ** P < 0.01; *** P < 0.001. Published OnlineFirst September 12, 2017; DOI: 10.1158/1078-0432.CCR-17-0901
highest binding affinity to FOXM1 with $\Delta G$ of $-12.94$ kcal/mol and FullFitness of $-1556.22$ kcal/mol compared to Thioistrepton (31) or other hit compounds (Table 1). This effect was translated on the ability of Mon to exert a superior inhibitory effect on FOXM1 transcriptional activity compared with Thioistrepton (Supplementary Fig. S3A). Importantly, Mon showed a dose-dependent inhibition on FOXM1 transcriptional activity (Fig. 3B) and FOXM1 expression at protein levels (Supplementary Fig. S3B). Mon has predominant selectivity for high FOXM1-expressing 42DENZR cells by reducing cell proliferation (Fig. 3C) and inducing apoptosis (Supplementary Fig. S3G).

To further gain insight into the molecular interactions between Mon and FOXM1, we conducted explicit solvent MD simulations. During 10 ns MD simulations, we observed that Mon was tightly bound to the DNA-binding region of FOXM1 throughout the simulation period, and Arg236, Tyr241, Lys278, and His287 residues of FOXM1 interact with Mon over 80% of the total MD simulation time using a frequent contact map (Fig. 3D). Importantly, the compound forms strong H-bond interactions with His287 and Tyr241, whereas Leu291, His287, and Trp281 residues make strong hydrophobic contacts with the chemical core (Fig. 3D). Using drug DARTS assay (24), we confirmed that Mon binds to FOXM1 in cell assay (Supplementary Fig. S3D). To further evaluate if Mon binds to FOXM1 DNA-binding region, in silico–guided site-directed mutagenesis was performed. Based on the contact frequency map as shown in Fig. 3D, two double mutants (R236A and Y241A; K278A and H287A) were generated that contribute to Mon binding to FOXM1 and were overexpressed in LNCaP cells. We found that Mon exhibited weaker effect on mon reduced FOXM1 binding to promoters of its target genes (<0.01 for R236A_Y241A or K278A_H287A, respectively; Fig. 3E) as well as on cell proliferation (P value < 0.05; Fig. 4B). Based on the above finding, we explored the Mon effect on the population of CD24$^+$/CD49b$^+$ cells, ALDH activity, and tumorspheres, as readouts of stem-like phenotype. First, we found that 42DENZR and 42FENZR were enriched with CD24$^+$/CD49b$^+$ cells, ALDH activity, and tumorspheres (Supplementary Fig. S4A) and exhibit high ALDH (Supplementary Fig. S5A) compared with CD24$^+$/CD49b$^+$ population (Fig. 4E; Supplementary Fig. S5D and S5E). In addition, Mon significantly reduced the number and size of 42DENZR and 42FENZR cell tumorspheres (Fig. 4F). These data suggest that Mon targets not only FOXM1 but also stem cell-like phenotypes.

<table>
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<th>Drug</th>
<th>Target</th>
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<th>FOXM1, FullFitness (kcal/mol)</th>
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<td>Monensin</td>
<td>Antibiotic</td>
<td>$-12.94$</td>
<td>$-1556.22$</td>
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<td>$-788.62$</td>
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In particular, PCS1-specific gene signature was also reported to be enriched with stem-like phenotype (14). We compared the subtype-specific genes of PCS1, PCS2, and PCS3 with embryonic stem cell core signatures and found that approximately one-third of the PCS1 genes belong to these signatures (33, 34), whereas PCS2 and PCS3 signatures did not display these genes (<1% of the genes listed; Fig. 4A). Our ENZR CRPC cells enriched with PCS1 were also significantly enriched with ECS (ES score of 0.44, P value < 0.01), whereas Mon significantly downregulates ECS (ES score, $-0.40$, P value < 0.05; Fig. 4B). Based on the above finding, we explored the Mon effect on the population of CD24$^+$/CD49b$^+$ cells, ALDH activity, and tumorspheres, as readouts of stem-like phenotype. First, we found that 42DENZR and 42FENZR were enriched with CD24$^+$/CD49b$^+$ population (Supplementary Fig. S4A) and exhibit high ALDH (Supplementary Fig. S5A) compared with 16$^{D}CRPC$. Targeting FOXM1 using Mon or siRNA reduces CD24$^+$/CD49b$^+$ population (Fig. 4C; Supplementary Fig. S4B and S4C) or ALDH activity (Fig. 4D; Supplementary Fig. S5B and S5C). Interestingly, ALDH$^{High}$ cells which display higher expression of FOXM1 (cells sorted from 42DENZR and 42FENZR cells using FACS) were more sensitive to Mon compared with ALDH$^{Low}$ cells (Fig. 4E; Supplementary Fig. S5D and S5E). In addition, Mon significantly reduced the number and size of 42DENZR and 42FENZR cell tumorspheres (Fig. 4F). These data suggest that Mon targets not only FOXM1 but also stem cell-like phenotypes.
Figure 4.

PCS1 and ENZ\textsuperscript{\textregistered} CRPC cells display stem-like features that are targeted by FOXM1 inhibition. A, The percentage of PCS1, PCS2, and PCS3 subtype-specific genes in embryonic stem cell signatures (33, 34). B, Mon effect on Wong and colleagues’ embryonic stem cell signature assessed using GSEA on ctrl- and Mon-treated 42DENZR cells. C, CD49\textsuperscript{high} population. Percentage of high CD49\textsuperscript{high} expressing cells in 42DENZR and 42FENZR cells in comparison with 16DENZR cells using flow cytometry (left). Effect of Mon on the percentage CD49\textsuperscript{high} population in 42DENZR and 42FENZR cells using flow cytometry (middle). Effect of FOXM1 siRNA on CD49\textsuperscript{high} population in 42DENZR and 42FENZR cells using flow cytometry (right). D, ALDH activity. Percentage of ALDH activity in 42DENZR and 42FENZR cells in comparison with 16DENZR cells assessed by Aldefluor ALDH activity using flow cytometry (left). Effect of Mon on ALDH activity in 42DENZR and 42FENZR cells in comparison with 16DENZR cells assessed by Aldefluor ALDH activity using flow cytometry (middle). Effect of FOXM1 siRNA ALDH activity in 42DENZR and 42FENZR cells in comparison with 16DENZR cells assessed by Aldefluor ALDH activity using flow cytometry (right). E, Relative confluence of 42DENZR and 42FENZR cells with high and low ALDH activity in response to Mon treatment assessed by IncuCyte (Essen Biosciences). Graphs represent pooled data from three independent experiments. F, Effect of Mon on the size and number of 42DENZR and 42FENZR tumorspheres.

\( P < 0.05; \quad P < 0.01; \quad P < 0.001.\)
Discussion

This preclinical study addresses the major clinical challenge of targeting the most aggressive subtype of prostate cancer PCs1 derived from 4,600 patient’s data (14). FOXM1 was found to be the most enriched master regulator pathway in “AR-indifferent” ENZ R CRPC cells that we identified as PCS1. Interestingly, we found that PCS1 signature is upregulated in “AR-indifferent” CRPC-neuroendocrine (NEPC) patients in the 2016 Beltran cohort (compared with CRPC-Adeno; Supplementary Fig. S7A; ref. 13) and in NPp53 abiraterone-exceptional nonresponder mice (compared with NPp53 vehicle) that transdifferentiate to neuroendocrine (Supplementary Fig. S7B; ref. 35) as well as in RB1, TP53, and PTEN double and triple knockout compared with wild type in the 2017 Ku data set (Supplementary Fig. S7C; ref. 36). These data suggest that PCS1 signature is found in a broad phenotype of aggressive prostate cancer. In agreement, our ENZ R cells exhibit a luminal B-like subtype by PAM50 classification (Pearson correlation 0.47, P values < 0.01), which is associated with the poorest clinical prognoses in prostate cancer (37). In addition, FOXM1 and its target genes were upregulated in high-risk prostate cancer and correlated with poor survival and was found to be increased in TP53Alt/RB1Alt phenotype characterized by aggressive NEPC features (36). Importantly, mitotic kinase AURKA and N-Myc that previously were linked to NEPC phenotype (13) are FOXM1 target genes (38, 39). Together, these findings further link the PCS1 signature to FOXM1 pathway, AR indifferent and/or neuroendocrine prostate cancer.

We discovered Mon as a novel FOXM1-binding agent with higher binding affinity to previously established FOXM1 inhibitor Thiostrepton (40). Mon targets and reduces FOXM1 pathway activity and reduces PCS1 signature. Mon selectively inhibits cell proliferation in high FOXM1-expressing cells in vitro and in vivo without any toxicity, induces apoptosis, and reduces self-renewal. These data are in accordance with previous reports indicating that FOXM1 regulates major hallmarks of cancer such as proliferation/cell cycle, metastasis, genomic instability, stem cell renewal, DNA damage repair, and drug resistance (29, 41–43). Together, these findings suggest that Mon is as a promising drug candidate to inhibit master regulator FOXM1 and PCS1 tumors.

In conclusion, our results reveal FOXM1 as a major pathway activated in PCS1 and ENZ R CRPC and Mon as a novel FOXM1 pathway activity, and ALDH activity in 42DENZR tumors in vivo.

**Figure 5.**

Mon reduces ENZ R CRPC xenograft growth, FOXM1 pathway, and ALDH activity in vivo. A, Effect of vehicle or Mon on tumor growth of 42DENZR xenografts. Graph represents pooled data from 6 vehicle- and 6 Mon-treated tumors. B, Relative mRNA expression assessed by qRT-PCR of FOXM1, CCNB1, and SKP2 in vehicle- vs. Mon-treated xenografts. C, Percentage of cells with high ALDH activity in 42DENZR tumor xenografts treated with vehicle or Mon. Graph represents pooled data from 4 vehicle- and 4 Mon-treated tumors. *, P < 0.05; **, P < 0.01.
and PCS1 subtype—targeting agent that also reduces stem-like phenotype in vitro and in vivo. High FOXM1 pathway expression also correlated with aggressive prostate cancer phenotype in prostate cancer patients including high Gleason score and poor survival. Because there is a lack of third-line treatment options for ENZ<sup>2</sup> CRPC, in the clinic and there are no therapies available for PCS1, our results indicate that targeting the FOXM1 pathway may provide a novel therapeutic strategy for this aggressive subset of prostate cancer associated with treatment resistance.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


