Title
Identification of Potential Therapeutic Targets in Advanced Prostate Cancer

Author
Yang, Nianxin

Publication Date
2022

Peer reviewed|Thesis/dissertation

Identification of Potential Therapeutic Targets in Advanced Prostate Cancer

By

NIANXIN YANG

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry, Molecular, Cellular and Developmental Biology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

_____

Hongwu Chen, Chair

_____

Kermit Carraway

_____

Allen Gao

Committee in Charge

2022

Table of Contents

Abstract

Identification of potential therapeutic targets in advanced prostate cancer

Metastatic castration-resistant prostate cancer (mCRPC) features high intratumoral cholesterol levels, due to aberrant regulation of cholesterol homeostasis. However, the underlying mechanisms are still poorly understood. The retinoid acid receptor-related orphan receptor gamma (RORγ), an attractive therapeutic target for cancer and autoimmune diseases, is strongly implicated in prostate cancer progression. We demonstrate in this study that in mCRPC cells and tumors, RORγ plays a crucial role in deregulation of cholesterol homeostasis. First, we found that RORγ activates the expression of key cholesterol biosynthesis proteins, including HMGCS1, HMGCR, and SQLE. Interestingly, we also found that RORγ inhibition induces cholesterol efflux gene program including ABCA1, ABCG1 and ApoA1. Our further studies revealed that liver X receptors (LXRα and LXRβ), the master regulators of cholesterol efflux pathway, mediate the function of RORγ in repression of cholesterol efflux. Finally, we demonstrated that RORγ antagonist in combination with statins has synergistic effect in killing mCRPC cells through blocking statin-induced feedback induction of cholesterol biosynthesis program and that the combination treatment also elicits stronger anti-tumor effects than either alone. Altogether, our work revealed that in mCRPC, RORγ contributes to aberrant cholesterol homeostasis by induction of cholesterol biosynthesis program and suppression of cholesterol efflux genes. Our findings support a therapeutic strategy of targeting RORγ alone or in combination with statin for effective treatment of mCRPC.

Treatment-induced neuroendocrine prostate cancer (tNEPC) is an advanced, aggressive, and treatment-resistant subtype of prostate cancer that has lost dependency on androgen receptor (AR) signaling and develops neuroendocrine traits. However, the mechanisms by which tNEPC cells survive and proliferate are still poorly understood. Histone deacetylases (HDACs) have been studied in various types of cancers, and a number of HDAC inhibitors has been developed and tested in clinical studies. In this study, we demonstrate that in tNEPC cells, HDACs play vital role in tNEPC cell survival and proliferation, while also possess potential anti-cancer functions. First, we found that HDAC inhibition blocks NEPC cell growth. Notably, HDAC pan-inhibitors and some specific HDAC-selective inhibitors display high potency in tNEPC cell growth inhibition. Next, we found that HDAC inhibitors downregulate cell cycle and cell survival related gene programs. Interestingly, some pro-metastasis and neuroendocrine development gene programs are induced by HDAC inhibitors. Finally, using specific HDAC-selective inhibitors, we identified several potential candidates that play essential roles in tNEPC cells, including HDAC1, HDAC3, HDAC4, and HDAC5. Together, our findings identify HDACs as potential therapeutic targets for tNEPC and point out that additional target-specific compounds with less non-intended side-effects need to be developed.

Acknowledgements

First and foremost, I am would like to say thank you to my major professor and supervisor, Dr. Hongwu Chen for your support, guidance, and advice, during my PhD career. You have shared valuable knowledge in the fields of advanced prostate cancer, epigenetic regulation, molecular biology, and cancer biology, which greatly broadened my sights into the fields. Your support and supervision significantly helped me during my PhD career and helped the completion of my dissertation.

Next, I would like to thank all my dissertation committee members, Dr. Hongwu Chen, Chair, Dr. Kermit Carraway, and Dr. Allen Gao for your support not only on my Qualifying Exam but also on my follow up studies. I really appreciate the time and effort you spent on my projects and the valuable suggestions and encouragements you provided.

I would also like to thank all my lab members, Yatian, Hongye, and Xiong. Thank you for your support and technical advices on my experiments and all the fun and precious time we spent in the past few years. You really made our lab felt like home to me.

Last but not least, I would like thank the Biochemistry, Molecular, Cellular and Developmental Biology Graduate Group for providing me the opportunity to study in such a great program. I really appreciate the great courses taught by all my respected professors, the friendly and supportive environment, and the abundant resources that the program provided. I also would like to thank my BMCDB academic advisor Dr. Daniel Starr for your guidance in my PhD career development.

Chapter 1

Deregulation of Cholesterol Homeostasis by a Nuclear Hormone Receptor Crosstalk in Advanced Prostate Cancer

1.1 Abstract

Metastatic castration-resistant prostate cancer (mCRPC) features high intratumoral cholesterol levels, due to aberrant regulation of cholesterol homeostasis. However, the underlying mechanisms are still poorly understood. The retinoid acid receptor-related orphan receptor gamma (RORγ), an attractive therapeutic target for cancer and autoimmune diseases, is strongly implicated in prostate cancer progression. We demonstrate in this study that in mCRPC cells and tumors, RORγ plays a crucial role in deregulation of cholesterol homeostasis. First, we found that RORγ activates the expression of key cholesterol biosynthesis proteins, including HMGCS1, HMGCR, and SQLE. Interestingly, we also found that RORγ inhibition induces cholesterol efflux gene program including ABCA1, ABCG1 and ApoA1. Our further studies revealed that liver X receptors (LXRα and LXRβ), the master regulators of cholesterol efflux pathway, mediate the function of RORγ in repression of cholesterol efflux. Finally, we demonstrated that RORγ antagonist in combination with statins has synergistic effect in killing mCRPC cells through blocking statin-induced feedback induction of cholesterol biosynthesis program and that the combination treatment also elicits stronger anti-tumor effects than either alone. Altogether, our work revealed that in mCRPC, RORγ contributes to aberrant cholesterol homeostasis by induction of cholesterol biosynthesis program and suppression of cholesterol efflux genes. Our findings support a therapeutic strategy of targeting RORγ alone or in combination with statin for effective treatment of mCRPC.

1.2 Introduction

Deregulated cholesterol homeostasis at stages such as synthesis, efflux, uptake, storage and metabolism is often associated with tumorigenesis and cancer progression[1-3]. Cholesterol is not only a crucial component of cell membrane but also a key regulator of cell signaling via its control of membrane fluidity and lipid rafts. Cholesterol is also the precursor of metabolites such as steroids, oxysteroids, bile acids, and certain vitamins. In advanced prostate cancer (PCa) such as metastatic castration-resistant PCa or mCRPC, tumors often feature high intratumoral cholesterol levels, due to aberrant regulation of cholesterol homeostasis[4, 5]. Notably, studies have demonstrated that both cholesterol biosynthesis and efflux are reprogrammed in mCRPC, which may be major contributors to tumor growth and lethal progression[6-8]. Indeed, elevated expression of cholesterol biosynthesis rate-limiting enzymes such as SQLE have been strongly correlated with poor outcome of PCa[7, 9, 10]. Low or loss of expression of key efflux proteins such as ABCA1 and metabolic enzymes such as CYP27A1 are also associated with the disease progression[6, 11]. Moreover, aggressive tumors often display elevated contents of cholesterol esters and specific cholesterol metabolites, which is associated with tumor growth, metastasis, and drug resistance[12-14]. Loss of expression and function of tumor suppressors such as Pten and p53 has been causally associated with aberrant tumor cholesterol homeostasis. Heightened signaling by PI3K-Akt and mTOR or the androgen receptor (AR) also contributes to cholesterol deregulation[4]. Despite of some recent progress, mechanisms underlying aberrant cholesterol homeostasis in advanced PCa are still poorly understood.

Cholesterol homeostasis is regulated chiefly at transcriptional level by two major groups of transcription factors. Sterol regulatory element-binding protein 1 and 2, particularly SREBP2, plays a pivotal role in activating cellular cholesterol biosynthesis genes such as HMGCS1 and

SQLE, in response to low level of cholesterol [1], whereas liver X receptors (LXRs), members of the nuclear receptor superfamily, regulate cholesterol efflux [2]. LXR target genes include major cholesterol efflux-related enzymes or transporters, including ABCA1, ABCG1 and ApoE[15-17]. Cholesterol-lowering drug statins have shown tumor suppressing activities in preclinical models[18]. However, their clinical trials have not shown significant benefit to PCa patients[19], likely due to tumor's feedback activities in cholesterol homeostasis, including SREBP2-mediated upregulation of cholesterol biosynthesis genes[19, 20]. Therefore, therapeutics that suppress elevated intratumoral cholesterol levels without induction of the feedback response are optimal strategies. Recently, drugs or compounds such as ezetimibe that target NPC1L1 protein for blocking cholesterol absorption and terbinafine that can inhibit SQLE were shown to be effective in inhibition of PCa tumor growth[9, 21], thus supporting the notion that targeting the tumor aberrant cholesterol homeostasis can be an attractive option.

RORγ and its immune cell-specific isoform RORγt, another member of the NR family, play important functions in control of tissue metabolism and immune response[3, 22]. Recently, a number of antagonists/inverse agonists of RORγ have been developed and several of them are at clinical trials for autoimmune disorders[3, 22-24]. Previous studies of us and others demonstrated that RORγ plays a crucial role in tumor growth and progression[3, 25, 26]. We also showed that small-molecule antagonists of RORγ such as XY018 and SR2211 are effective in blocking mCRPC cell and xenograft tumor growth[25]. In addition, our recent study identified RORγ as an essential activator of the entire cholesterol-biosynthesis program in triple negative breast cancer (TNBC), dominating the function of SREBP2[26]. Here, we report that in mCRPC, RORγ acts both as an essential activator of cholesterol biosynthesis program and a major suppressor of cholesterol efflux program. RORγ antagonists effectively abolish statin-induced feedback

regulation and inhibit tumor growth. Therefore, our findings suggest that RORγ is a new player of cholesterol homeostasis deregulation in PCa.

1.3 Materials and Methods

Cell Culture

C4-2B and 22Rv1 prostate cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Gemini or Hyclone). Cells were grown at 37 °C in 5% CO2 incubators. 22Rv1 cell line was obtained from American Type Culture Collection (ATCC), and C4-2B cell line was obtained from UroCor Inc. (Oklahoma City, OK). Cells lines were tested being negative for mycoplasma regularly.

Chemicals

XY018 (Purity > 99%) was synthesized by WuXi AppTec. SR2211 (Purity > 98%) was obtained from TOCRIS. All statin and other compounds were obtained from Sigma-Aldrich, Selleck, or MedChemExpress.

Cell Viability and Growth Assays

For cell viability, cells were seeded in 96-well plates at 2000 cells per well in a total of 100 µl of media. Serially diluted small molecule compounds in 100 µl of media were added to each designated well after 24h. After 4 days of treatment, culture media was removed and 50 µl of Cell-Titer GLO reagents (Promega) was added, and then luminescence was measured on Varioskan Lux multimode microplate reader (ThermoFisher Scientific). All experimental points were measured as triplicates, and the experiments were repeated at least two or three times. The

luminescence of cells treated with vehicle was set at 100% viability, and all other data were standardized to percentage of viable cells.

Measurement of Cholesterol Content in Cells

22Rv1 cells were cultured in 6-well plates and treated with compounds for 48 or 72h. After treatment, cells were digested off the plates by 0.5% trypsin in PBS and collected into 1.5 ml Eppendorf tubes. Cellular cholesterol was extracted with organic solvants (7:11:0.1, chloroform/isopropanol/Triton X-100). Cholesterol level of each cell extract was measured with AmplexTM Red Cholesterol Assay Kit (ThermoFisher Scientific). Florescent readings were normalized to protein concentrations. All measurements were repeated three times, and the whole experiments were repeated for at least two or three times.

Cholesterol Rescue Assay

C4-2B and 22Rv1 cells were seeded in 6-well plates for 24h. The cells were randomly assigned into three groups, with each group treated with either vehicle (DMSO), or RORγ antagonists XY018 (5 μM) or SR2211 (5 μM) for 48h. Each group of cells were also separated into three sub-groups, with each sub-group receiving cholesterol supplement (Sigma-Aldrich, C4951) in its designated quantity (0, 1,25, or 2,5 μg/ml medium). After treatment, all cells were collected and the cell numbers were counted with Countess 3 Automated Cell Counter (ThermoFisher Scientific) following the manufacturer's instructions. All measurements were set as duplicates, and the whole experiments were repeated for at least three times.

qRT-PCR and Immunoblotting Analysis

Total RNA was isolated and purified from cells seeded in 6-well plates or from xenograft tumors. cDNA was reverse-transcribed using qScriptTM cDNA SuperMix (Quanta Biosciences, 95048),

and then amplified and measured with 2X SYBRGreen qPCR Mastermix (Bimake, B21202) or PowerUpTM SYBRTM Green Master Mix (ThermoFisher Scientific, A25742). The SYBR fluorescence values were collected, and the melting-curve was analyzed. Expression of each transcript was normalized by GAPDH as the internal reference, and expression change in folds was calculated. The experiments were performed at least two to three times, with internal duplicates or triplicates, and the data was presented either in heat maps or as mean values ± s.d. Sequences of the primers are listed in Table S1.1 Cell lysates were analyzed by immunoblotting with antibodies recognizing indicated proteins. Details of the antibodies are listed in Table S1.2

RNA-seq and Data Analysis

22Rv1 cells were treated with vehicle, XY018 (1.25 or 5 μM), Simvastatin (1.25 or 5 μM), Atorvastatin (1.25 or 5 μM), or the combination of XY018 and each statin (1.25 μM each) for 48h before RNA extraction. C4-2B cells were treated as previously described. RNA-seq libraries from 1 μg of total RNA were prepared using Illumina Tru-Seq RNA Sample Prep Kit according to the manufacturer's instructions. Libraries were validated with Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Sequencing was performed by Illumina HiSeq 2000 sequencer at BGI Tech (Hong Kong). The FASTQ-formatted sequence data were analyzed by using a standard BWA-Bowtie-Cufflinks workflow. Sequence reads were mapped to human-genome assembly (GRCh37/hg19) with BWA and Biotite software. Cufflinks package was utilized for transcript assembly, quantification of normalized gene and isoform expression in RPKM (reads per kilobase per million mapped reads) or FPKM (Fragments per kilobase of exon model per million mapped reads), and analysis of differential expression.

siRNA Transfection

siRNAs for gene knockdown were purchased from Thermofisher (NR1H3 siRNA, s14685; NR1H2 siRNA, s19568) or Santa Cruz Biotechnology (LXRα siRNA, sc-38828; LXRβ siRNA, sc-45316). Transfections were performed with DharmaFECT#1 (Dharmacon) or Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer's instruction in OptiMEM (Invitrogen). For RNA extraction, cells were treated with siRNAs for 48h, and for protein extraction, cells were treated with siRNAs for 72h.

Mouse Models and Treatments

Four-week-old male mice (strain: NOD.CB17-Prkdc^{scid}/NCrHsd) were purchased from Envigo (Indianapolis, IN). Mice were housed under standard conditions, under a 12-hour light/12-hour dark cycle. For 22Rv1 cell line-derived xenograft, 2 × 106 cells were suspended in a total of 100 µl PBS and Matrigel (1:1) mixture, and implanted subcutaneously into the dorsal flank on both sides of the mice. For xenograft tumor growth curve analysis, when the tumor volumes were approximately 50 mm^3, mice were randomized and then administered with 100 µl of vehicle (intraperitoneally (i.p.), in 15% Cremophor EL, Calbiochem, 82.5% PBS, and 2.5% DMSO), RORγ antagonists XY018 (5 mg/kg, i.p., in 15% Cremophor EL, Calbiochem, 82.5% PBS, and 2.5% DMSO), simvastatin (25 mg/kg orally, in PBS), or a combination of XY018 and simvastatin (by their respective dose and administration method). Tumor volumes were monitored every three days by using calipers with volume calculated by using the equation: $\pi/6$ (length × width^2). Body weight was also monitored during the treatment period. At the end of the study, mice were sacrificed, and tumors were dissected and weighed. For tumor gene expression analysis, when the tumor volumes reached approximately 200 mm^3, mice were treated with vehicle (i.p.), XY018 (25 mg/kg, i.p.), simvastatin (25 mg/kg, orally), or a combination of XY018 and simvastatin (by their

respective dose and administration method) for 7 days. At the end of the treatment period, mice were sacrificed, and tumors were collected and subjected to RNA extraction.

The animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis.

Statistical Analysis

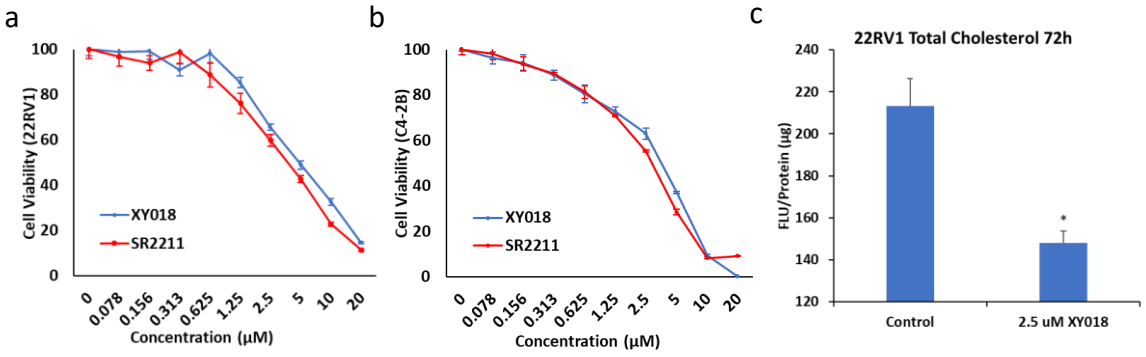
All statistical details of experiments are included in the figure legends or the specific Method sections. The data are presented as mean values ± s.d. Statistical analysis was performed using two tailed Student's t tests to compare the means. $P < 0.05$ was considered to be statistically significant.

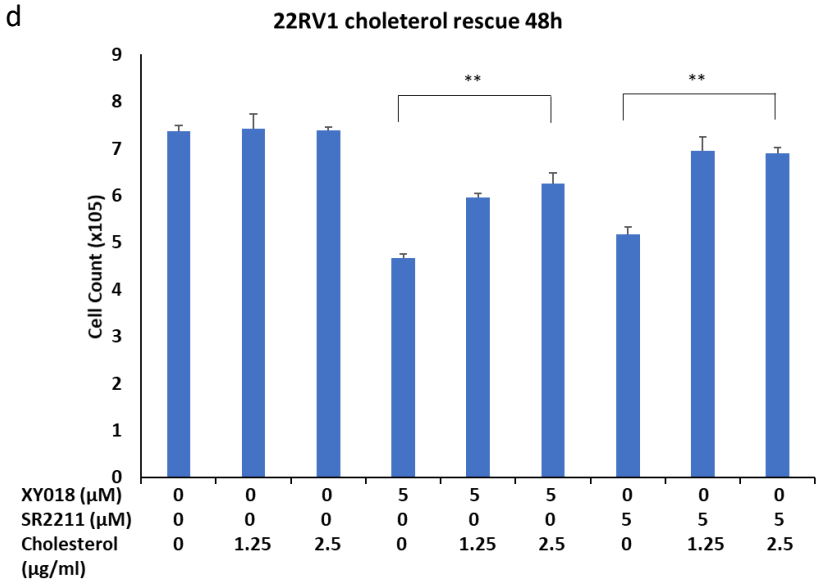
1.4 Results

1.4.1 RORγ antagonists inhibit mCRPC cell growth and survival by decreasing intracellular cholesterol levels

Our previous studies showed that RORγ is overexpressed and plays a crucial role in mCRPC tumors[25, 27]. As demonstrated in our previous studies[25, 27, 28], small molecule antagonists (also known as inverse aganists) of RORγ such as XY018 or SR2211 potently inhibited the growth and survival of mCRPC cells (Fig 1.1a, b). Given that elevated cholesterol levels promote PCa cell growth and survival, we thus investigated whether the effect of RORγ inhibition is linked to its potential function in control of the increased cholesterol level in mCRPC. Thus, we measured cellular cholesterol levels in 22Rv1 cells treated with RORγ antagonist XY018 using a commercially available assay kit. Consistent with the cell viability test, RORγ antagonist XY018 significantly reduced cellular cholesterol levels in 22Rv1 cells (Fig. 1.1c). To examine whether the reduction of cellular cholesterol levels contributes to the growth inhibition effect of the RORγ antagonists, we performed a cholesterol rescue experiment. Indeed, the RORγ antagonist-induced

growth inhibition was largely mitigated by the exogenous cholesterol supply in both mCRPC cells (Fig. 1.1d, e, Fig. S1.1a, b). Therefore, these results suggest that RORγ antagonists inhibit mCRPC cell growth and survival at least in part through decreasing cellular cholesterol levels. They also suggest that RORγ plays an important role in control of high cholesterol levels in mCRPC.


a
Cell Viability (22RV1)
XY018
SR2211
Concentration (μM)
b
Cell Viability (C4-2B)
XY018
SR2211
Concentration (μM)
c
22RV1 Total Cholesterol 72h
FLU/Protein (μg)
Control
2.5 uM XY018


d
22RV1 choleterol rescue 48h
Cell Count (x105)
XY018 (μM)
SR2211 (μM)
Cholesterol (μg/ml)

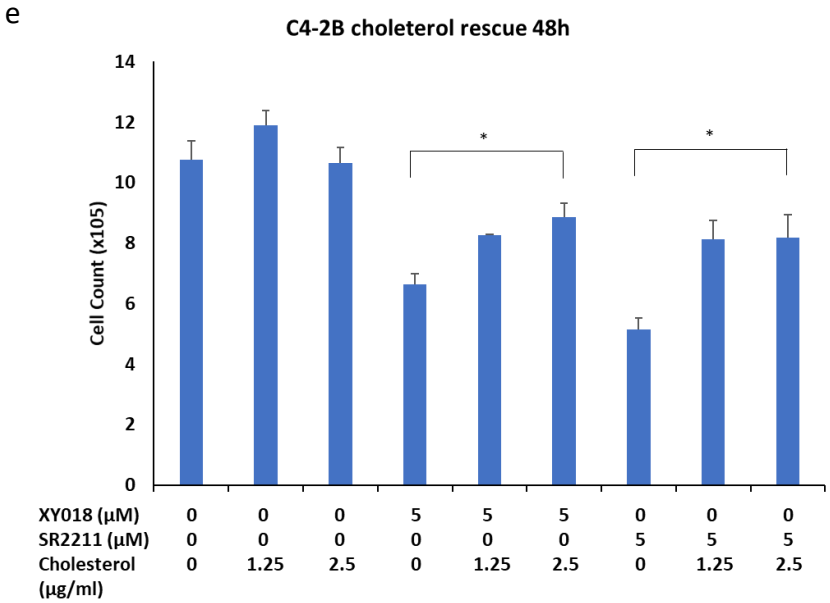

e
C4-2B choleterol rescue 48h
Cell Count (x105)
XY018 (μM)
SR2211 (μM)
Cholesterol (μg/ml)

Figure 1.1 RORγ antagonists inhibit mCRPC cell growth and survival by decreasing intracellular cholesterol levels. (**a-b**) Cell Vialibility, measured by Cell-Titer GLO (Promega) of 22Rv1 (**a**) and C4-2B (**b**) cells treated with the indicated concentration of RORγ antagonists XY018 and SR2211 for 4 d. (**c**) Total cholesterol levels in relative florescent units/protein, measured by Amplex^{TM} Red Cholesterol Assay Kit of 22Rv1 cells treated with indicated concentration of XY018 for 72 h. (**d-e**) Cell numbers of 22Rv1 (**d**) and C4-2B (**e**) cells treated with indicated concentration of XY018, SR2211, and cholesterol for 48 h. Data are shown as mean ± s.d. n = 3. Student's t test. $*p < 0.05$, $**p < 0.01$.

1.4.2 RORγ controls expression of key cholesterol biosynthesis enzymes in mCRPC cells

In TNBC, RORγ can function as a master activator of cholesterol biosynthesis[26]. To examine whether in mCRPC, RORγ plays a similar function, we treated 22Rv1 and C4-2B cells with antagonist XY018 and performed qRT-PCR to analyze the changes in expression of the 21 enzyme encoding genes in cholesterol biosynthesis program. The analysis demonstrated that over half of the cholesterol biosynthesis genes are significantly downregulated by RORγ antagonist XY018 in both cell lines, including the rate-limiting enzymes HMGCR and SQLE, which are often upregulated in PCa tumors (Fig. 1.2a). To further examine the effect of RORγ function inhibition on gene expression, we treated 22Rv1 cells with a low dose and a high dose of XY018 and performed RNA-seq analysis. Again, we found that over half of the cholesterol biosynthesis genes are downregulated by RORγ antagonist XY018 in a dose-dependent manner (Fig. 1.2b). Consistently, XY018 treatment significantly downregulated protein expression of key cholesterol biosynthesis enzymes in both cell lines, including HMGCS1, HMGCR, SQLE, and DHCR24 (Fig. 1.2c, d). In addition, we examined the effect of RORγ inhibition in androgen-responsive prostate cancer cell LNCaP and found similar but less dramatic inhibition in the gene expression (Fig. S1.1c). To validate that RORγ functions to activate cholesterol biosynthesis program, we treated the CRPC cells with RORγ specific siRNAs or RORγ agonists SR0987 and LYC55716[29, 30].

Consistent with the results from the antagonists, siRNA treatments significantly downregulated protein expression of key cholesterol biosynthesis enzymes in 22RV1 cells (Fig. S1.2a), while RORγ agonist treatments enhanced the protein expression in both 22RV1 and C4-2B cells (Fig. S1.2b, c). Together, these results suggest that RORγ functions as a major activator of cholesterol biosynthesis program in mCRPC.

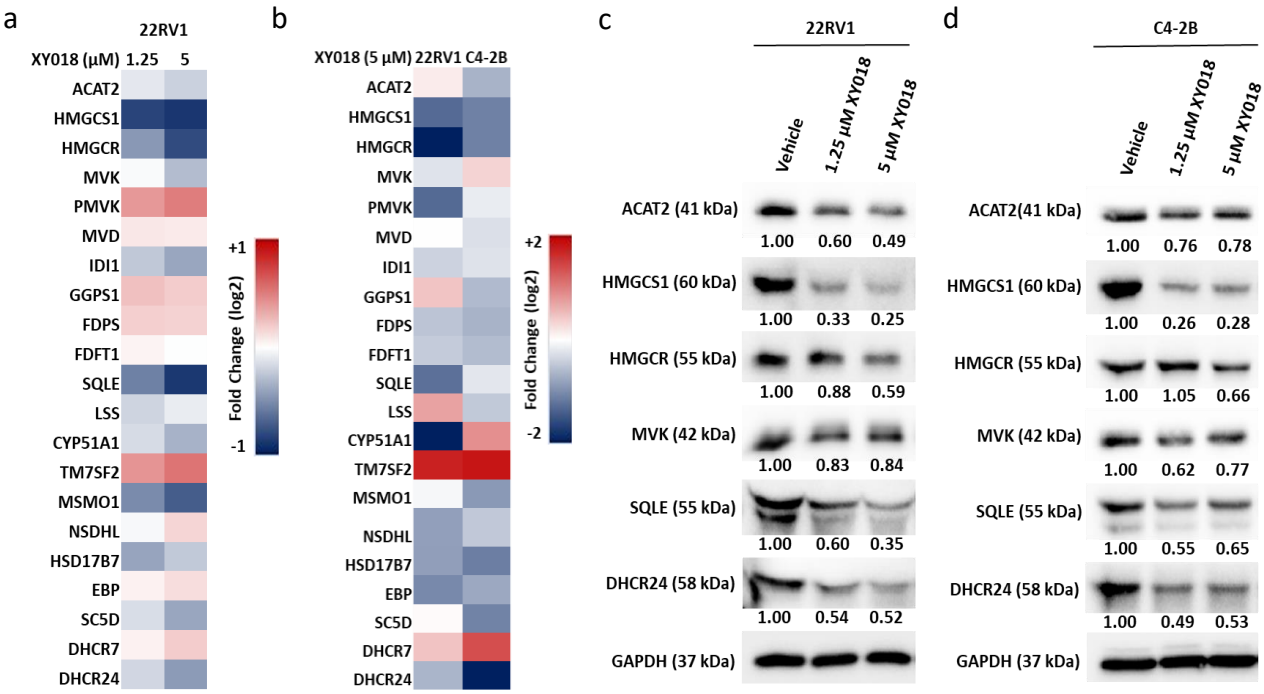


Figure 1.2 RORγ antagonists inhibit key cholesterol biosynthesis gene expression in mCRPC cells. (**a**) Heat map of mRNA expression of 21 cholesterol biosynthesis genes, as measured by qRT-PCR in 22Rv1 and C4-2B cells treated with 5 μM of XY018 for 48 h, as compared to vehicle (DMSO), n = 3. (**b**) Heat map of mRNA expression changes of 21 cholesterol biosynthesis genes, as detected by RNA-seq in 22Rv1 cells treated with indicated concentrations of XY018 for 48 h, as compared to vehicle (DMSO). (**c-d**) Immunoblotting of proteins involved in cholesterol biosynthesis pathway in 22Rv1 (**c**) and C4-2B (**d**) cells treated with indicated concentrations of XY018 for 72 h.

1.4.3 Inhibition of RORγ stimulates cholesterol efflux gene program in mCRPC cells

Aberrant cholesterol homeostasis in PCa involves both elevated biosynthesis and reduced efflux of cholesterol[26, 31]. To further examine the potential role of RORγ in control of other aspects of cholesterol homeostasis, we analyzed expression changes in all cholesterol homeostasis related genes in the RNA-seq data of 22Rv1. Surprisingly, we found that expression of cholesterol efflux related genes such as ABCA1, ABCG1, ABCG8, APOA1, -A5, APOE, LRP1 and NPC2 is significantly elevated by RORγ antagonist XY018 in 22Rv1 cells (Fig. 1.3a). The three ATP binding cassette transporters are directly responsible for cellular cholesterol efflux. APOA and APOE are crucial components of lipoproteins that are responsible for cholesterol packaging and transport. Upon comparing the RNA-seq data from the culture of two different mCRPC cell lines (22Rv1 and C4-2B) treated with antagonist XY018, we found that gene programs involved in reverse cholesterol transport and cholesterol efflux are among the most highly enriched in the 671 commonly upregulated transcripts by XY018 in both 22Rv1 and C4-2B cell lines (Fig. 1.3b). Our qRT-PCR analysis confirmed that ABCA1, ABCG1, MYLIP, APOA5, APOE, LRP1 and NPC2 are strongly induced by the RORγ antagonist in the two mCRPC cells (Fig. 1.3c). Given the key role played by ABCA1 and ABCG1 in cholesterol efflux in PCa tumors, we analyzed their protein expressions in both 22Rv1 and C4-2B cells and found that RORγ antagonist XY018 significantly increased their protein levels (Fig. 1.3d, e). In addition, siRNA knockdown of RORγ also enhanced the expression of ABCG1 in 22RV1 cells (Fig. S1.2a), while treatments of cells with RORγ agonists SR0987 and LYC55716 inhibited the expression of both ABCA1 and ABCG1 in 22RV1 and C4-2B cells (Fig. S1.2b, c). Therefore, these results suggest that in PCa, RORγ functions to suppress the expression of cholesterol efflux program and that targeting of RORγ with the

inhibitors induces the efflux gene expression, which likely contributes to the overall effect of the inhibitors in reduction of cellular cholesterol level in PCa tumor cells.

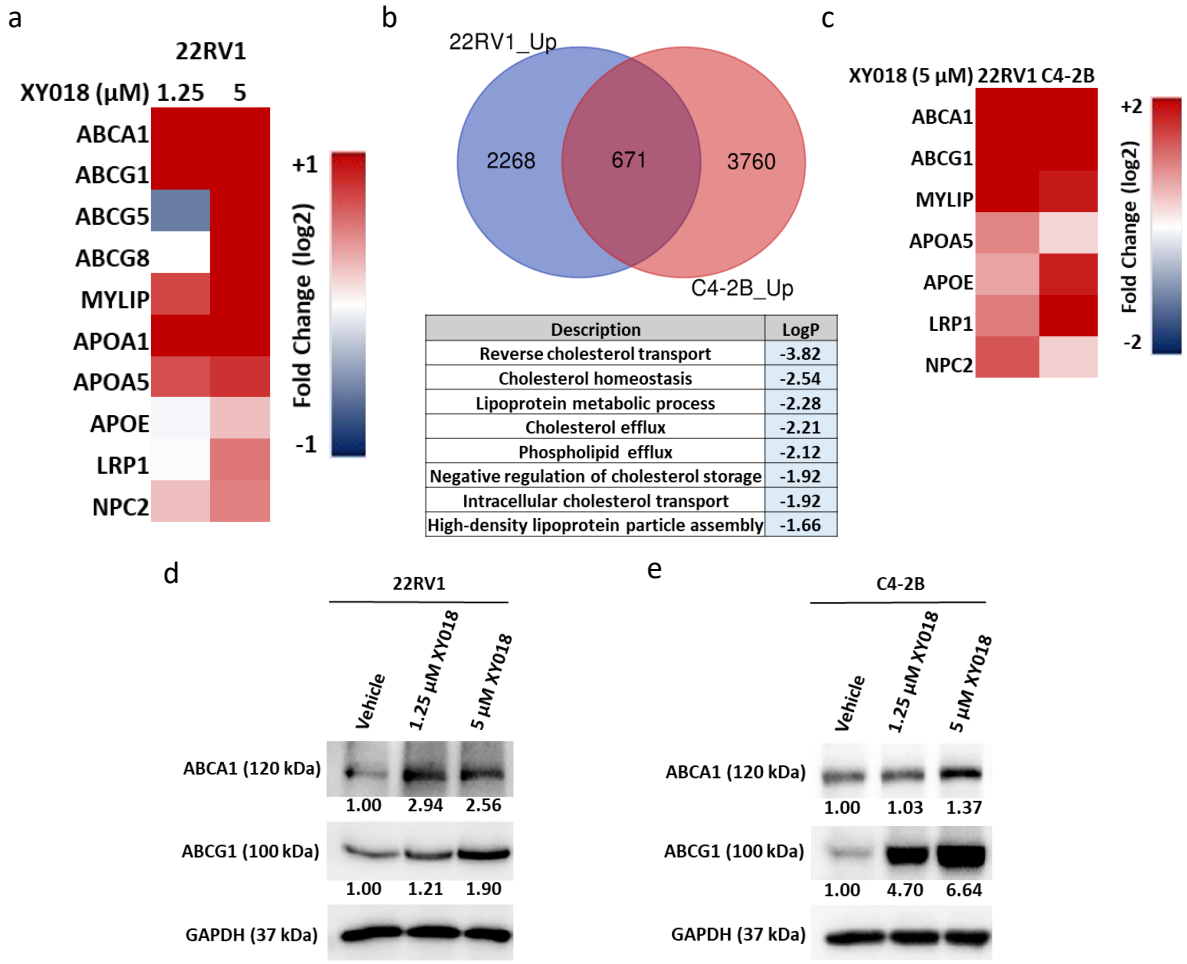


Figure 1.3 RORγ antagonists stimulate cholesterol efflux gene expression in mCRPC cells. (**a**) Heat map of mRNA expression changes of 10 cholesterol efflux genes, as detected by RNA-seq in 22Rv1 cells treated with indicated concentrations of XY018 for 48 h, as compared to vehicle (DMSO). (**b**) Venn diagram of the number of genes with expression significantly upregulated (1.3-fold), as detected by RNA-seq of 22Rv1 and C4-2B cells treated with 5 μM of XY018 for 48 h (top). Gene ontology analysis of the 671 genes with expression upregulated in both 22Rv1 and C4-2B cells treated with XY018 as shown in the top part (bottom). (**c**) Heat map of mRNA expression changes of 7 cholesterol efflux genes, as measured by qRT-PCR in 22Rv1 and C4-2B cells treated with 5 μM of XY018 for 48 h, as compared to vehicle (DMSO), n = 3. (**d-e**) Immunoblotting of proteins involved in cholesterol efflux pathway in 22Rv1 (**d**) and C4-2B (**e**) cells treated with indicated concentrations of XY018 for 72 h.

1.4.4 LXRs mediate the regulation of cholesterol efflux program by RORγ in mCRPC

RORγ is a well characterized transcriptional activator[32, 33], which does not readily explain the induction effect of its antagonist on the cholesterol efflux genes. On the other hand, LXRα and LXRβ (with gene name NR1H3 and NR1H2 respectively) are well known master regulators of cholesterol efflux[15-17]. Thus, we examined whether the effect of RORγ antagonism on cholesterol efflux gene program is via LXRs. qRT-PCR analysis revealed that RORγ antagonist XY018 treatment significantly enhanced LXRβ/NR1H2 gene expression in both 22Rv1 and C4-2B cells while slightly increased LXRα/NR1H3 gene expression in both cell lines (Fig. 1.4a, b). In addition, the expressions of both LXRs along with the cholesterol efflux gene program were significantly elevated by RORγ antagonist XY018 treatment in androgen responsive LNCaP cells (Fig. S1.1d). Consistently, both LXRα and LXRβ protein expression were elevated by XY018 treatment in the mCRPC cells (Fig. 1.4c, d). Moreover, RORγ specific siRNA treatments enhanced LXRβ protein expression in 22RV1 cells (Fig S1.2a), while RORγ agonists SR0987 and LYC55716 treatments inhibited both LXRα and LXRβ protein expression in 22RV1 and C4-2B cells (Fig S1.2b, c). Next, to determine whether the RORγ antagonist effect on cholesterol efflux is through LXR, we treated the cells with RORγ antagonist XY018 and LXRα/β-specific siRNAs. LXRα and LXRβ siRNAs effectively knocked down the expression of their respective target proteins in both cell lines (Fig. 1.4e, f). In 22Rv1 cells, single treatment of siLXRα and siLXRβ and their combination treatment were all effective to significantly abolish the induction of ABCA1 and ABCG1 genes by XY018 (Fig. 1.4g, h). On the other hand, in C4-2B cells, single treatment of siLXRα and siLXRβ showed relatively moderate effect on the expression induction by XY018, while their combination treatment significantly abolished the expression induction (Fig. 1.4i, j).

Together, these results indicate that the function of RORγ in suppression of cholesterol efflux gene program is through its positive regulation of LXR expression in mCRPC cells.

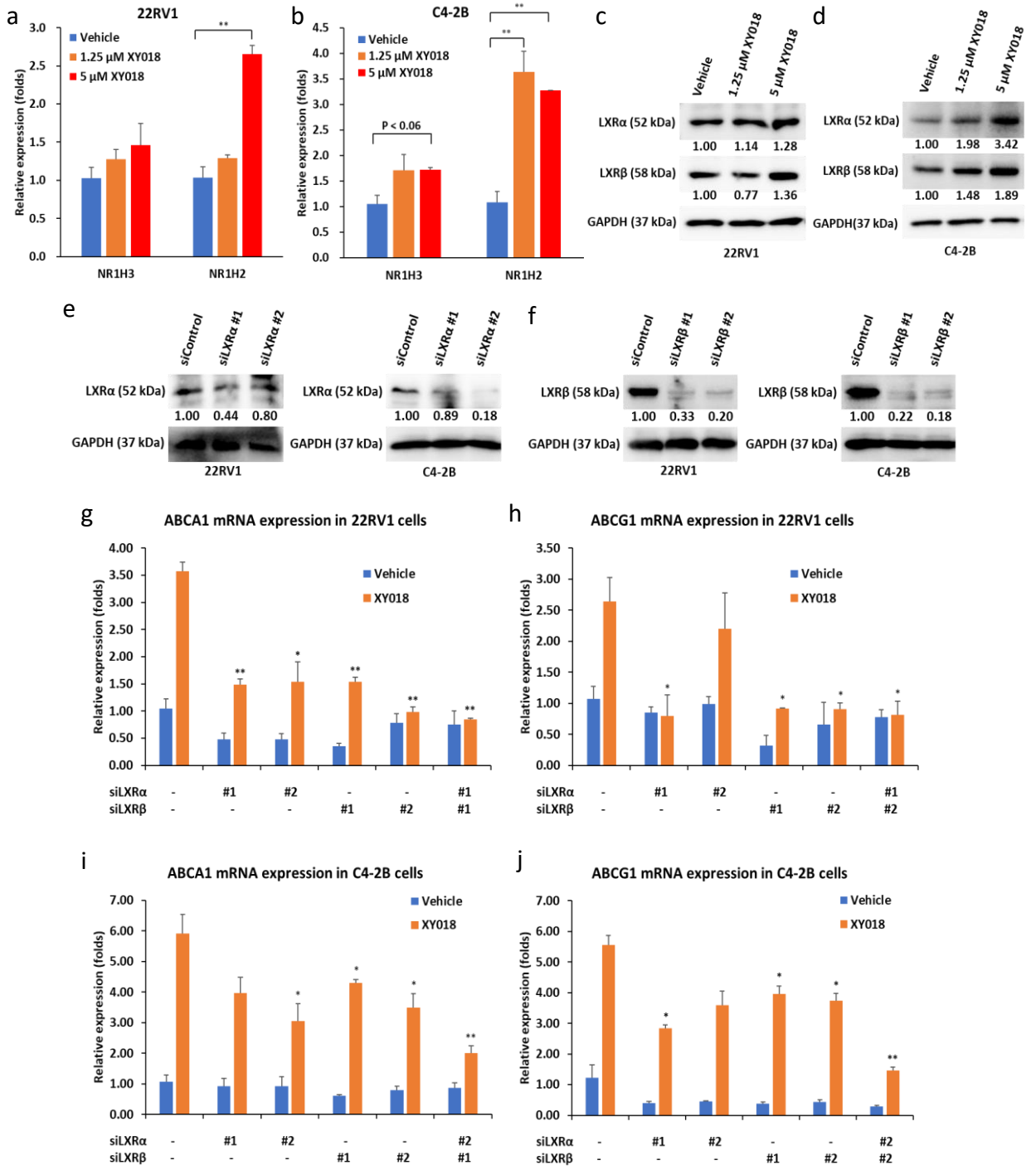


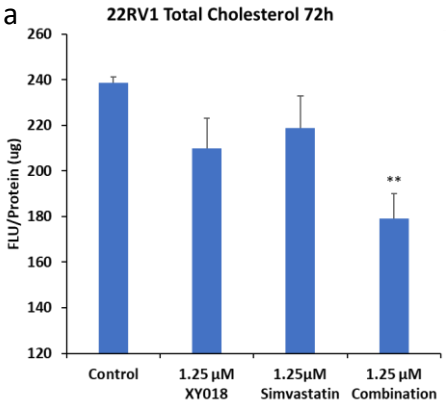
Figure 1.4 LXR expression regulates aberrant cholesterol efflux gene program mediated by RORγ in mCRPC cells. (**a-b**) Relative expression of NR1H3 and NR1H2 mRNA in fold changes, as detected by qRT-PCR in 22Rv1 (**a**) and C4-2B (**b**) cells treated with indicated concentrations of XY018 for 48 h. (**c-d**) Immunoblotting of LXRα and LXRβ proteins in 22Rv1 (**c**) and C4-2B (**d**) cells treated with indicated concentrations of XY018 for 72 h. (**e-f**) Immunoblotting of LXRα (**e**) and LXRβ (**f**) proteins in 22Rv1 and C4-2B cells treated with indicated siRNAs for 48 h. (**g-j**) Relative expression of ABCA1 and ABCG1 mRNA in fold changes, as detected by qRT-PCR in 22Rv1 (**g, h**) and C4-2B (**i, j**) cells treated with vehicle (DMSO) or 2.5 μM XY018 and indicated siRNAs for 48 h. The experiments were repeated three times. Data are shown as mean ± s.d. n = 3. Student's t test. $*p < 0.05$ $**p < 0.01$.

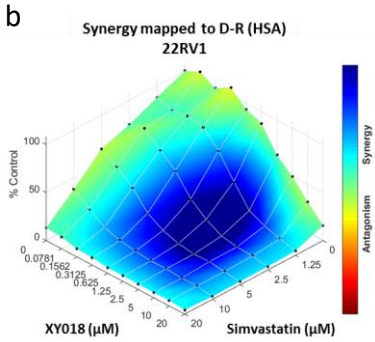
1.4.5 RORγ inhibition synergizes with statins in killing mCRPC cells through abolishing statin-induced feedback

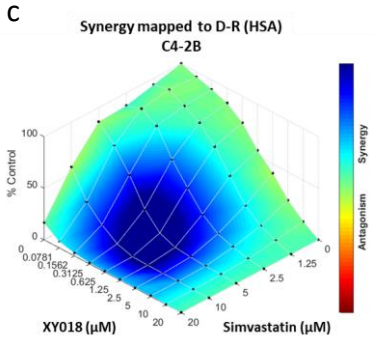
Statins are widely used as cholesterol lowering drugs[34, 35]. Despite of promising results from preclinical studies, statins have not shown remarkable benefits to advanced PCa patients in clinical trials[19]. As shown in Fig. 5A, treatment of mCRPC cells with a relatively low concentration (1.25 μM) of either RORγ antagonist XY018 or simvastatin did not elicit a significant decrease of cellular cholesterol level. However, their combination significantly reduced the cholesterol content (Fig. 1.5a). Remarkably, when combined with XY018, the widely used statins such as simvastatin (SMV), atorvastatin (ATV), fluvastatin (FLV) and pitavastatin (PTV) all possessed prominent synergistic effect in growth inhibition on both CRPC cell lines, indicating that the growth inhibition synergy with the RORγ antagonist is not limited to a specific statin drug (Fig. 1.5b, c, Fig. S1.3 a1-3, b1-3).

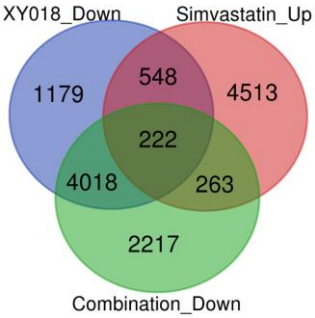
Induction by statin of a feedback, up-regulation of cholesterol biosynthesis program and consequently rebound of tumor cholesterol level is postulated to be a major reason underlying the lack of efficacy at the clinic[1, 19]. Thus, to examine whether statin elicits a similar feedback

mechanism in mCRPC cells, we performed RNA-seq analysis of 22Rv1 cells treated with simvastatin and atorvastatin. Our gene ontology analysis of the altered gene expression revealed that among the 1885 transcripts upregulated by both statins (Fig. S1.3c), cholesterol biosynthesis related programs are among the most highly enriched (Fig. S1.3d). To examine the impact of RORγ antagonists on statin-induced feedback, we performed RNA-seq profiling of cells treated by both simvastatin and XY018. Comparing the altered gene expressions in cells treated by the statin alone, XY018 alone or their combination revealed a significant overlap of 222 genes that are up-regulated by statin and down-regulated by either XY018 alone or XY018 in combination with simvastatin (Fig. 1.5d). Gene ontology analysis of the 222 genes showed that cholesterol and isoprenoid biosynthetic processes are the most significantly altered gene programs (Fig. 1.5e), therefore indicating that when used in combination, RORγ antagonist can strongly mitigate the feedback induction of cholesterol biosynthesis programs by statin. Indeed, RNA-seq and qRT-PCR analysis demonstrated that treating cells with the RORγ antagonist not only abolished the statin induction of cholesterol biosynthesis genes but also resulted in a net decrease of the gene program (Fig. 1.5f, g). Remarkably, the combination treatment not only abolished statin-induced increase of cholesterol biosynthesis enzyme proteins, such as HMGCS1, HMGCR, SQLE, and DHCR24, but also resulted in a net decrease in their expression in both 22Rv1 and C4-2B cell lines (Fig. 1.5h, i). Together, these results suggest that RORγ inhibition can synergize with statins in killing mCRPC cells and that one mechanism is that the inhibition effectively abolishes statin-induced feedback induction of cholesterol biosynthesis program.



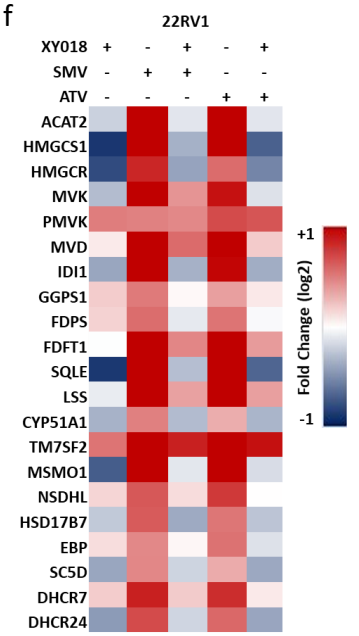


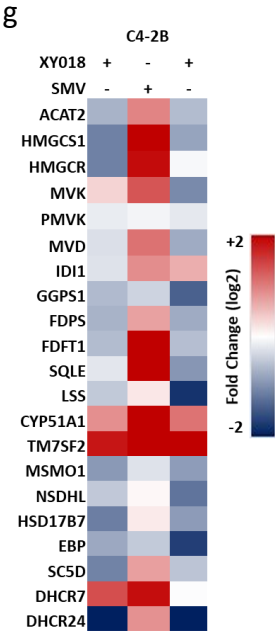


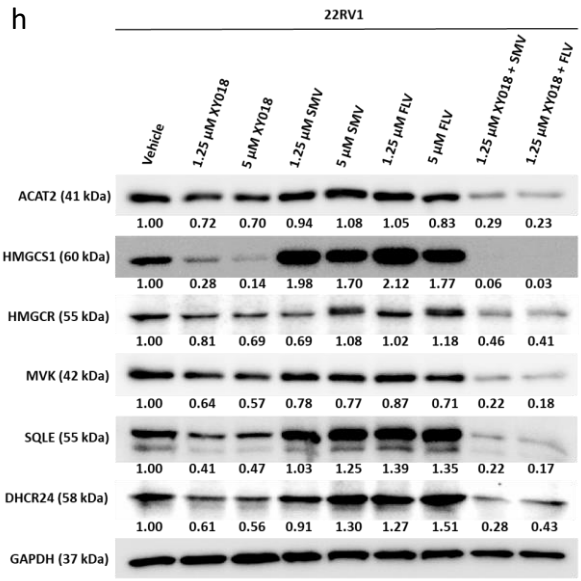


e

Description	LogP
Cholesterol biosynthetic process	-4.4
Regulation of catalytic activity	-2.6
Isoprenoid biosynthetic process	-2.2
Oxidation-reduction process	-1.8
Microtubule cytoskeleton organization	-1.6
Demethylation	-1.5
Cholesterol biosynthetic process via lathosterol	-1.5
Cholesterol biosynthetic process via desmosterol	-1.5
Glycosyl compound metabolic process	-1.5
Lipid metabolic process	-1.3







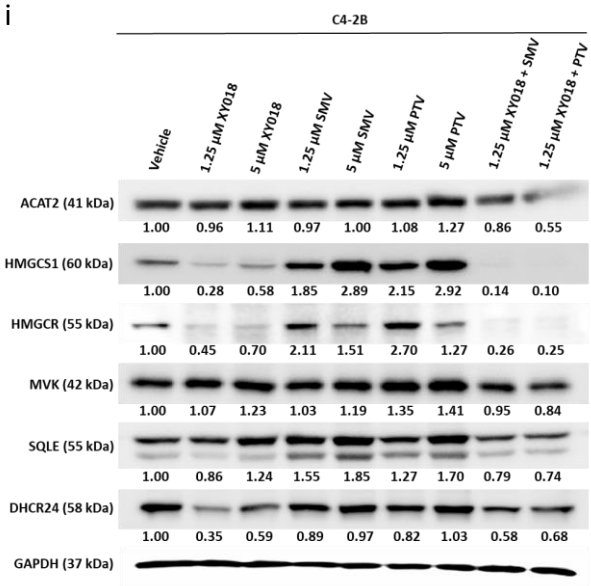


Figure 1.5 RORγ antagonists possess synergism with statins in killing mCRPC cells and abolish statin-induced feedback activities in cholesterol biosynthesis gene program. (**a**) Total cholesterol levels in relative florescent units/protein, measured by Amplex™ Red Cholesterol Assay Kit of 22Rv1 cells treated with indicated concentration of XY018, Simvastatin, or their combination for 72 h. (**b-c**) Drug combination synergism maps of 22Rv1 (**b**) and C4-2B (**c**) cells treated with XY018 and SMV as indicated concentration for 4 d. Blue indicates synergy while red indicates antagonism between drugs. (**d**) Venn diagram of the number of genes with expression significantly downregulated by XY018 (5 μM), or upregulated by SMV (5 μM), or downregulated by XY018 + SMV combination in 22Rv1 cells treated for 48 h, which are detected by RNA-seq. as detected by RNA-seq of 22Rv1 and C4-2B cells treated with 5 μM of XY018 for 48 h (top). (**e**) Gene ontology analysis of the 222 genes overlapped in expression alterations as shown in (**d**) in response to indicated compound treatment. (**f**) Heat map of mRNA expression changes of 21 cholesterol biosynthesis genes, as detected by RNA-seq in 22Rv1 cells treated with indicated concentrations of XY018 (5 μM), SMV (5 μM), ATV (5 μM), or XY018 + SMV/ATV combination (1.25 μM) for 48 h, as compared to vehicle (DMSO). (**g**) Heat map of mRNA expression changes of 21 cholesterol biosynthesis genes, as measured by qRT-PCR in C4-2B cells treated with XY018 (5 μM), SMV (5 μM), or XY018 + SMV combination (1.25 μM) for 48 h, as compared to vehicle (DMSO), n = 3. (**h-i**) Immunoblotting of proteins involved in cholesterol biosynthesis pathway in 22Rv1 (**h**) and C4-2B (**i**) cells treated with indicated concentrations of XY018, SMV, FLV, PTV or combinations of XY018 + statins for 72 h. Data are shown as mean ± s.d. n = 3. Student's t test. $^{**}p < 0.01$.

1.4.6 Targeting RORγ in combination with statin strongly inhibits mCRPC tumor growth through reprogramming cholesterol homeostasis

Knowing that RORγ antagonists and statins have synergistic effect in killing mCRPC cells, we next evaluated the therapeutic potential of the combination treatment in 22Rv1 xenograft tumor model. Intraperitoneal administration of a relatively low dose (5 mg/kg) of RORγ antagonist XY018 alone or oral administration of simvastatin (25 mg/kg) alone significantly inhibited the tumor growth by around 40% in tumor size. Notably, their combined treatment showed a

significantly stronger tumor inhibition than either alone (Fig. 1.6a). Tumor weights were also measured and were consistent with tumor sizes (Fig. 1.6b). Moreover, no significant change in the animal body weight was observed over the course of the treatment (Fig. 1.6c). Consistent with the results from 22Rv1 cells, tumor cholesterol biosynthesis gene expression was significantly inhibited by XY018 and its combination with statin. Importantly, tumor cholesterol efflux genes such as ABCA1, ABCG1 and the master regulator LXRs were also induced by XY018 treatment (Fig. 1.6d, e). Together, these results suggest that RORγ antagonist alone or its combination with statin can be effective in inhibition of mCRPC tumor growth and that down-regulation of cholesterol biosynthesis program and up-regulation of cholesterol efflux genes are the underlying mechanisms.

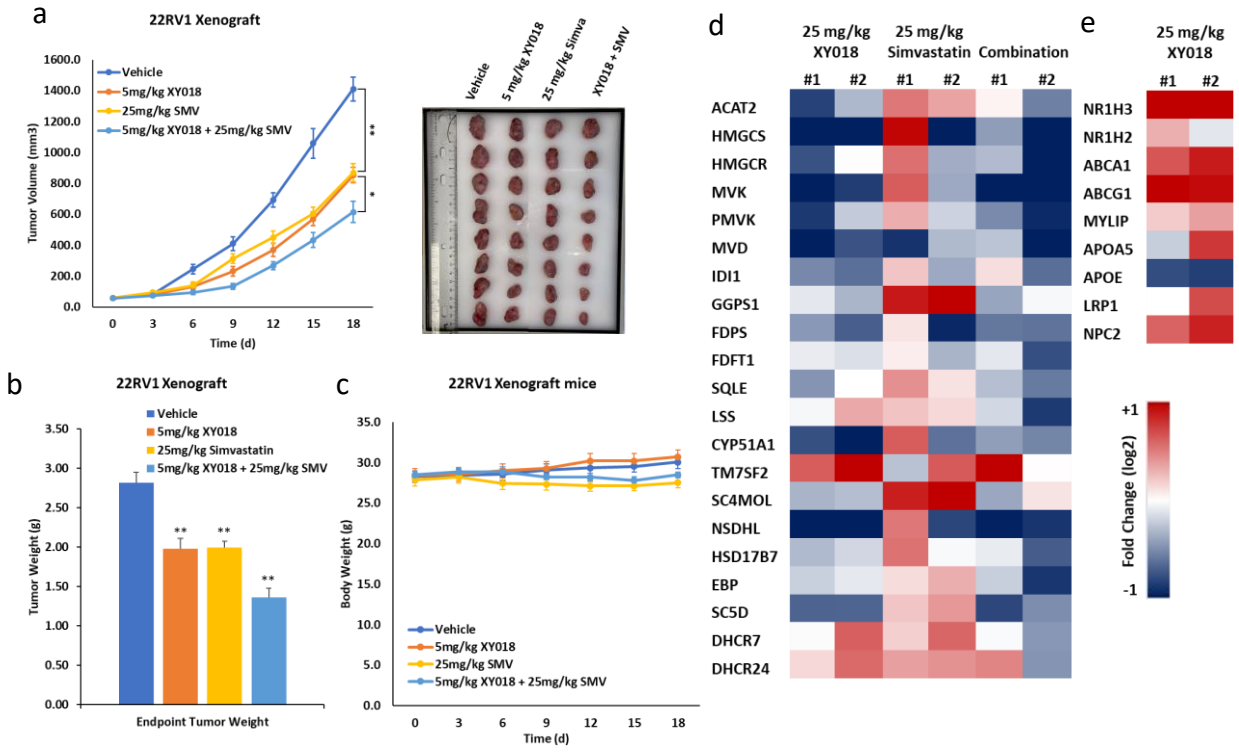


Figure 1.6 RORγ antagonist in combination with statin inhibit mCRPC tumor growth. (**a**) Growth inhibition effect of the indicated treatments (XY018, 5 mg/kg i.p. daily; SMV, 25 mg/kg orally daily; the combination of the two treatments; or vehicle) on 22Rv1 xenograft tumors (n = 7 mice per group). Representative images are shown. (**b**) 22Rv1 xenograft tumors were dissected

and weighed at the end point of growth inhibition experiments from (**a**). (**c**) Mice bearing the xenograft tumors were weighed at the end point of the growth inhibition experiments from (**a**). (**d-e**) Heat maps of mRNA expression changes of 21 cholesterol biosynthesis genes (**d**) and 9 cholesterol efflux genes (**e**) as measured by qRT-PCR in the xenograft tumors treated with XY018 (25 mg/kg i.p. daily), SMV (25 mg/kg orally, daily), or XY018 + SMV combination for 7 d, as compared to vehicle. Data are shown as mean ± s.d. Student's t test. $*p < 0.05$ $**p < 0.01$.

1.5 Discussion

It is well established that tumors of advanced PCa feature a highly elevated cholesterol content. Previous studies have focused on the deregulation of cholesterol biosynthesis pathway and revealed aberrant expression and function of key enzymes such as SQLE. However, the mechanisms of how the different aspects of cholesterol homeostasis such as cholesterol efflux and biosynthesis are coordinately deregulated in the tumor are much less understood. In this study, we demonstrated that RORγ not only activates the expression of cholesterol biosynthesis program but also suppresses the expression of key cholesterol efflux genes, which include the major transporters such as ABCA1 and ABCG1 and APOA1. Therefore, our study identified, for the first time, RORγ as a unique transcriptional regulator that coordinately de-regulates the programs of cholesterol biosynthesis and efflux.

RORγ was identified to play a major role in PCa due to its prominent function in activating AR gene expression and enhancing AR function in driving PCa progression[25]. Later studies by us and others showed that RORγ also plays important roles in breast cancer, pancreatic cancer and small cell lung cancer through stimulating gene programs of metabolism, cancer stemness, proliferation, EMT, drug resistance and lineage fate[26, 36, 37]. Like its T cell isoform RORγt, tumor cell RORγ acts primarily as a potent transcriptional activator. Indeed, in TNBC, RORγ interacts with SREBP2 to hyper-stimulate cholesterol biosynthesis program. In this study, we

observed a similar function of RORγ in up-regulation of cholesterol biosynthesis, including the effective abolishment of statin-induced, SREBP2-mediated feedback mechanism by the antagonist of RORγ. Therefore, it is likely that a mechanism similar to the one in TNBC is responsible for the RORγ function in control of PCa cholesterol biosynthesis program. Interestingly, however, in PCa, we observe that RORγ suppresses the expression of LXRα and β, the two master regulators of cholesterol efflux program, which then leads to the suppression of the efflux genes. This is rather unique in that instead of acting as an activator, RORγ acts as a repressor to silence the two LXR genes. It is also uncommon that a member of the NR family such as RORγ controls the expression of the other NR members. Nevertheless, these observations underscore the central role played by RORγ in control of cholesterol homeostasis in advanced PCa. Currently, it is unclear how RORγ acts to silence LXR genes. Elucidation of the mechanism will likely take integrated approaches to identify the binding site of RORγ at the LXR genes and the co-factors involved.

Although both RORs and LXRs are considered as sensors of specific lipids, LXRs generally play a tumor-suppressive role in several types of cancer, including prostate cancer[3, 15, 38]. Although our current study is focused on the role of RORγ in control of LXR-mediated cholesterol efflux program, it is possible that their crosstalk may also occur at other pathways such as cell cycle, apoptosis, and oncogenic kinase signaling which appear to be the targets of LXR agonists[38]. Interestingly, RORγ and LXRs are both major players in tumor immune microenvironment. LXRs are key regulators of the functions of macrophage and other tumor-infiltrated immune cells such as myeloid-derived suppressor cells (MDSCs). Therefore, future studies are warranted to further dissect the interplays between RORγ and LXRs in tumor cells and tumor microenvironment.

In PCa, many studies made the link of statin use to a reduced risk of disease progression such as PSA-based biochemical recurrence (BCR) and poor survival. However, clinical trials with statins

have all failed to demonstrate a strong efficacy in treating advanced prostate cancer. Many factors likely contribute to the failure, which include statin-induced feedback activities of cholesterol homeostasis in the tumor, inter- and intra-tumor heterogeneity, lack of efficacy-indicating biomarker, and clear understanding of lipid metabolism in the disease including the impact of circulating cholesterol such as hypercholesterolemia and other lipids on the tumor[4, 39-41]. In our current and previous studies, we found that antagonists of RORγ can potently abolish the statin-induced, feedback up-regulation of cholesterol biosynthesis program, thus suggesting that targeting RORγ can be a better therapeutic strategy. However, we recognize that the significance of our studies is limited by the pre-clinical models we used. Further work with more clinically relevant models and clinical studies are needed to address the limitations and to further support the rationale of targeting RORγ and to provide new insights into the role of RORγ in control of lipid metabolism in the tumor cells, the tumor microenvironment and the host.

Elevated cholesterol biosynthesis likely stimulates the disease progression through several means, including androgen production to sustain AR activation and function[42-46]. Recent studies have identified several cholesterol biosynthesis intermediates and cholesterol metabolites as RORγ agonists[47-49]. It is thus tempting to speculate that RORγ induction of elevated cholesterol levels can further enhance its own functions in a feedforward manner for hyper-activating AR and RORγ itself in driving the disease progression. Therefore, development of new antagonists of RORγ that are highly effective, either alone or in combination with other therapeutics such as statins or ezetimibe will be an attractive strategy for treatment of mCRPC.

1.6 Supplementary Materials

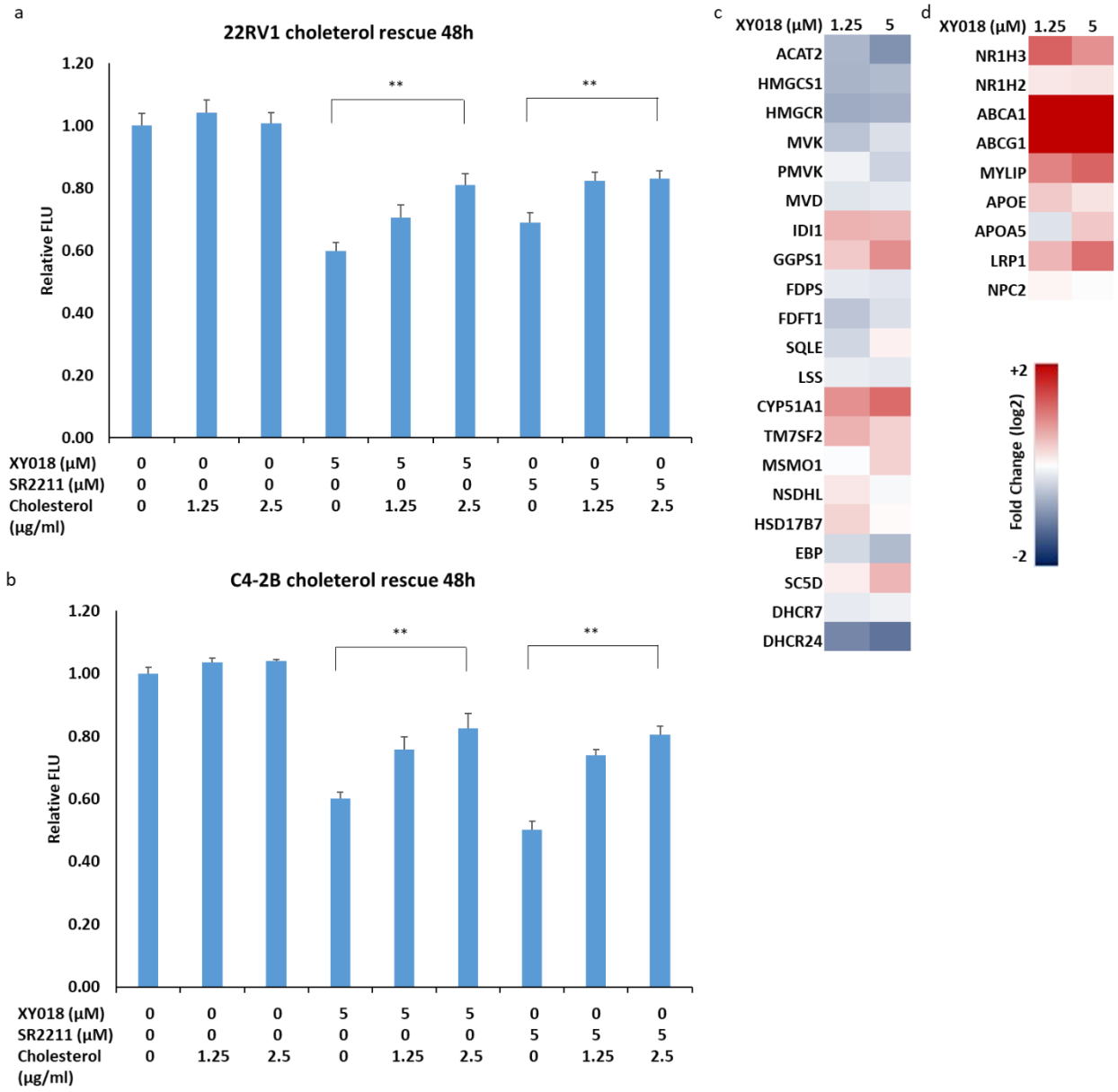


Figure S1.1 (**a and b**). **Exogenous cholesterol rescued growth inhibition by RORγ antagonists in mCRPC cells.** Cell viability was measured by CellTiter-Glo Assay of 22Rv1 (**a**) and C4-2B (**b**) cells treated with indicated concentration of XY018, SR2211, and cholesterol for 48 h. Data are shown as mean ± s.d. n = 3. Student's t test. ∗p < 0.05, ∗∗p < 0.01. (**c and d**). **RORγ antagonist inhibit the expression of cholesterol biosynthesis genes and enhances the expression of cholesterol efflux genes in androgen-responsive LNCaP cells.** Heat maps display mRNA expression of 21 cholesterol biosynthesis genes (**c**) or 7 cholesterol efflux genes

(**d**) measured by qRT-PCR in LNCaP cells treated with indicated concentrations of XY018 for 48 h, as compared to vehicle (DMSO). n = 3.

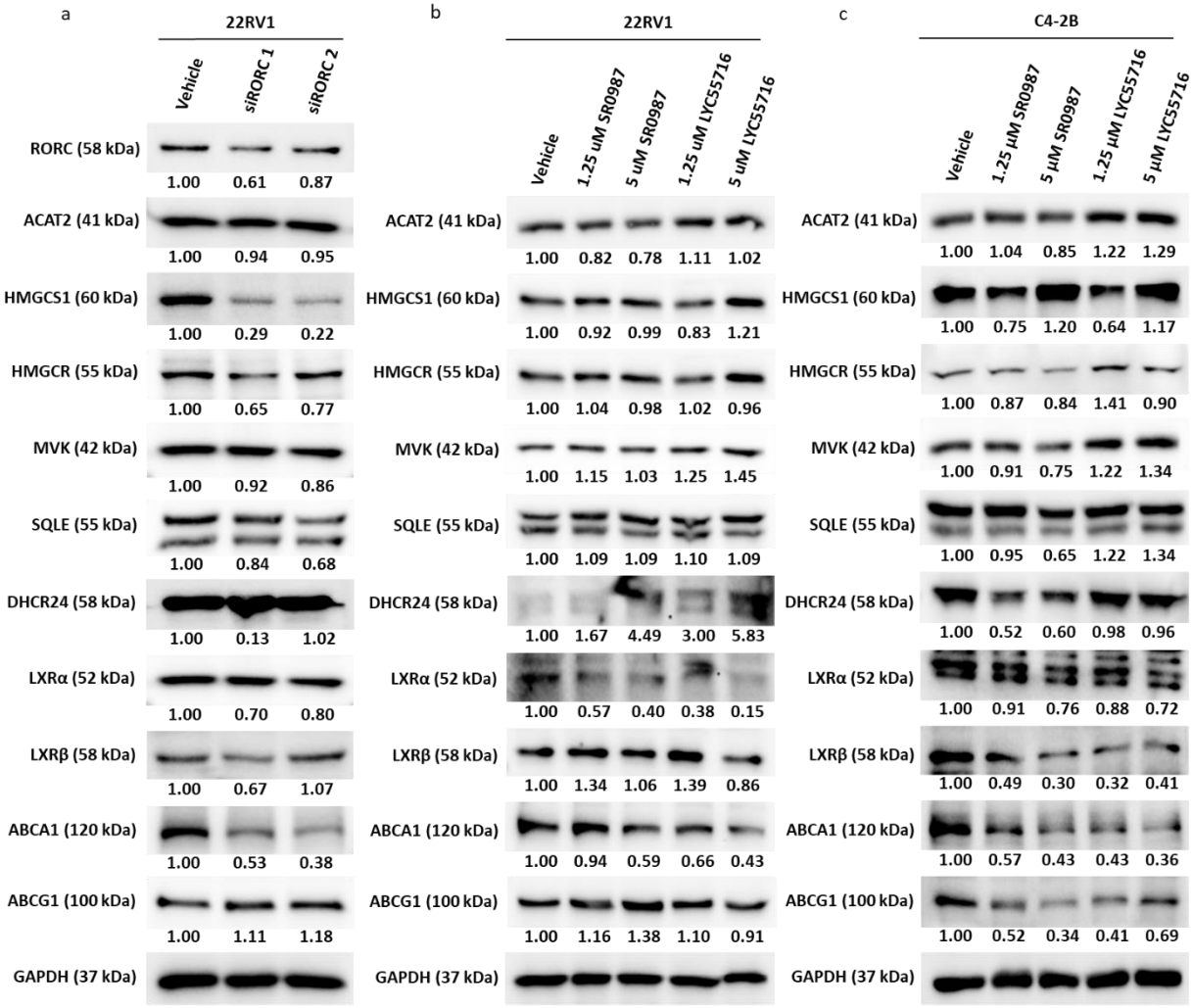
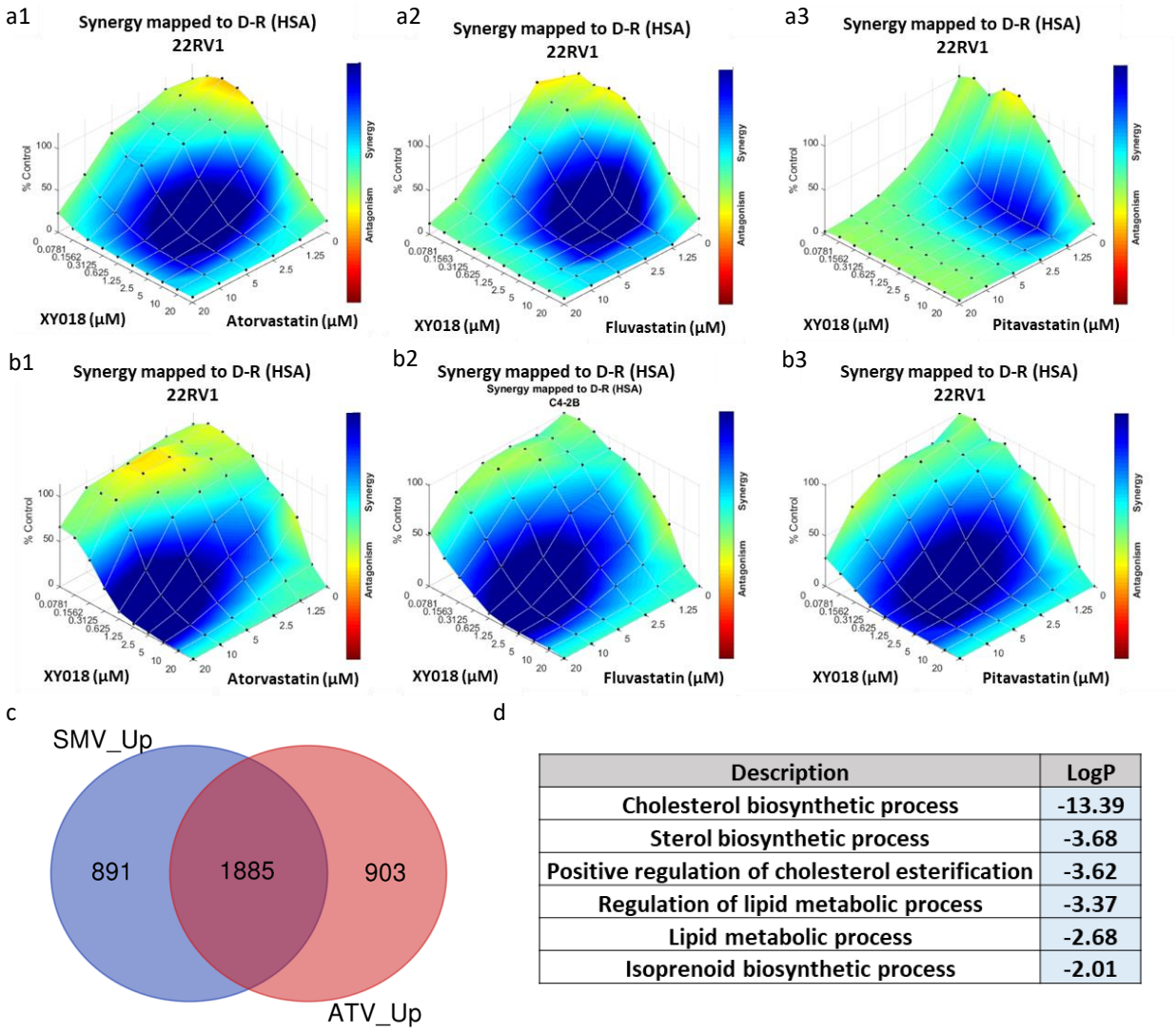


Figure S1.2 (**a**). **Knockdown of RORγ inhibits the expression of key cholesterol biosynthesis proteins and enhances the expression of key cholesterol efflux proteins.** Immunoblotting of RORγ and proteins involved in cholesterol biosynthesis and cholesterol efflux pathways in 22Rv1 cells treated with indicated siRNAs for 72 h. (**b and c**). **RORγ agonist treatment show effects oppositive to that of its antagonists on the protein expressions in mCRPC cells.** Immunoblotting of proteins involved in cholesterol biosynthesis and cholesterol efflux pathways in 22Rv1 (**b**) and C4-2B (**c**) cells treated with indicated concentrations of SR0987 and LYC55716 for 72 h.



c

d

Description	LogP
Cholesterol biosynthetic process	-13.39
Sterol biosynthetic process	-3.68
Positive regulation of cholesterol esterification	-3.62
Regulation of lipid metabolic process	-3.37
Lipid metabolic process	-2.68
Isoprenoid biosynthetic process	-2.01

Figure S1.3 (**a and b**). **RORγ antagonists possess synergism with statins in killing mCRPC cells.** Drug combination synergism maps of 22Rv1 (**a 1-3**) and C4-2B (**b 1-3**) cells treated with XY018 and ATV, FLV, or PTV at indicated concentrations for 4 days. Blue indicates synergy while red indicates antagonism between drugs. (**c and d**). **Statins induced feedback promotes cholesterol biosynthesis gene program expression.** Venn diagram of the number of genes with expression significantly upregulated (1.4-fold) in 22Rv1 treated with SMV (5 μM) and ATV (5 μM) for 48 h, as detected by RNA-seq (**c**). Gene ontology analysis of the 1885 genes with expression upregulated by both SMV and ATV in 22Rv1 cells (**d**).

Table S1.1 Primers and their sequences used in qRT-PCR

Cholesterol	Sequence	Cholesterol	Sequence
ACAT2_F	GCAGGTGTTCCTTCAATGG	NR1H3_F	CAGATCCGCCTGAAGAAACT
ACAT2_R	CACAGCTTTTAGGCCTGAC	NR1H3_R	TTAGCATCCGTGGGAACATC
HMGCS1_F	AGCTCAGAGAGGACACCCA	NR1H2_F	ACAGCGGCTCAAGAACTAATG
HMGCS1_R	GGTACTTTCTTGGCAGGGC	NR1H2_R	CGATCTCCTGGACTGAGATGAT
HMGCR_F	CCCAGCCTACAAGTTGGAA	ABCA1_F	GGAGCTGTTCACCGACAATAAG
HMGCR_R	GCTCCCATCACCAAGGAGT	ABCA1_R	CCCACCAAGTCCCAAGATAATG
MVK_F	GCTCAAGTTCCCAGAGATC	ABCG1_F	GGTGGTCTCGCTGATGAAA
MVK_R	ATGGTGCTGGTTCATGTCA	ABCG1_R	AATCTGCTGGGTTGTGGTAG
PMVK_F	CGGAGAGTGTCTGACATCC	MYLIP_F	GTGGAGCCTCATCTCATCTTAC
PMVK_R	AAGTTGTCCAGGCCACATT	MYLIP_R	GCACAGAGCTCCTCATAGTTAT
MVD_F	AGGACAGCAACCAGTTCCA	APOA5_F	GAAAGGTGTGGGCTGTGATA
MVD_R	GTGTCGTCCAGGGTGAAGA	APOA5_R	AAGTCTAGGCTTCAACTTGGG
IDI1_F	TGTTCCCTGCGAAAGGTAT	APOE_F	TCACAGGCAGGAAGATGAAG
IDI1_R	TGAACCTGTTGCTTGTCGA	APOE_R	AGCGCAGGTAATCCCAAA
GGPS_F	ACACGGTGAAACCCTGTCT	LRP1_F	GATAGCCAACTCCCAGAACATC
GGPS_R	AGAGGCACTATCTCGGCTC	LRP1_R	CTCATCCACGAAGCCCTTTAG
FDPS_F	AGGGCAATGTGGATCTTGT	NPC2_F	CCGAGCTTGGAACTTCGTTAT
FDPS_R	GAAAGAACTCCCCCATCTC	NPC2_R	GGTGACATTGACGCTGTAAGA
FDFT1_F	GGTCCCGCTGTTACACAAC		
FDFT1_R	AAAACTCTGCCATCCCAAT		
SQLE_F	GGCCATCTTTTGTTGGAGA		
SQLE_R	TTCAGAAGGGAATGGGAGT		
LSS_F	TTCCTGAGGCTCTCACAGG		
LSS_R	CCCTCCATCTGGATTTCTCA		
CYP51A1_F	GCTCAGTTGTTCCCTGCTTC		
CYP51A1_R	AAAATTAGCCAGGCATGGT		
TM7SF_F	CGCTTTCATCTTCAGCCTCT		
TM7SF_R	GCTCTGCCTCCTTCATCAAC		
MSMO1_F	ACTCTGTCTCCTTGGCTGG		
MSMO1_R	CATCGTGAAACCCCATCTC		
NSDHL_F	CTCAGCCAGTCACTCCTTC		
NSDHL_R	CTGCCTGCTTCAAGAAATC		
HSD17B7_F	CGTAGGACTTCCGAAAGCA		
HSD17B7_R	AGACAGCTTCTGCCTTGCT		
EBP_F	GCCTCAGCACCTAAGACTG		
EBP_R	ATGAACCCACACACTGCAA		
SC5D_F	TATCTCTTCCGCCCATGTTC		
SC5D_R	TGGCTCATTCACCATTTCA		
DHCR7_F	CATTGACATCTGCCATGAC		
DHCR7_R	ACAGGTCCTTCTGGTGGTT		
DHCR24_F	TGTTGCCTGAGCTTGATGA		
DHCR24_R	GACCAGGGTACGGCATAGA		

Table S1.2 Antibodies used in immunoblotting

Antibody	Vendor	Catalog #	Host Species
ACAT2	Abcam	ab131215	Rabbit
HMGCS1	Santa Cruz Biotechnology	sc-166763	Mouse
HMGCR	Santa Cruz Biotechnology	sc-271595	Mouse
MVK	Proteintech	12228-1-AP	Rabbit
SQLE	Santa Cruz Biotechnology	sc-271651	Mouse
DHCR24	Santa Cruz Biotechnology	sc-398938	Mouse
LXRα	Active Motif, Inc.	61175	Rabbit
LXRβ	Active Motif, Inc.	61177	Rabbit
ABCA1	Santa Cruz Biotechnology	sc-58219	Mouse
ABCG1	Proteintech	13578-1-AP	Rabbit
GAPDH	Cell Signaling Technology	14C10	Rabbit

1.7 References

1. Mullen, P. J., R. Yu, J. Longo, M. C. Archer, and L. Z. Penn. "The Interplay between Cell Signalling and the Mevalonate Pathway in Cancer." *Nat Rev Cancer* 16, no. 11 (2016): 718-31.
2. Luo, J., H. Yang, and B. L. Song. "Mechanisms and Regulation of Cholesterol Homeostasis." *Nat Rev Mol Cell Biol* 21, no. 4 (2020): 225-45.
3. Zou, H., N. Yang, X. Zhang, and H. W. Chen. "Rorγ Is a Context-Specific Master Regulator of Cholesterol Biosynthesis and an Emerging Therapeutic Target in Cancer and Autoimmune Diseases." *Biochem Pharmacol* 196 (2022): 114725.
4. Škara, L., A. Huđek Turković, I. Pezelj, A. Vrtarić, N. Sinčić, B. Krušlin, and M. Ulamec. "Prostate Cancer-Focus on Cholesterol." *Cancers (Basel)* 13, no. 18 (2021).
5. Hager, M. H., K. R. Solomon, and M. R. Freeman. "The Role of Cholesterol in Prostate Cancer." *Curr Opin Clin Nutr Metab Care* 9, no. 4 (2006): 379-85.
6. Lee, B. H., M. G. Taylor, P. Robinet, J. D. Smith, J. Schweitzer, E. Sehayek, S. M. Falzarano, C. Magi-Galluzzi, E. A. Klein, and A. H. Ting. "Dysregulation of Cholesterol Homeostasis in Human Prostate Cancer through Loss of Abca1." *Cancer Res* 73, no. 3 (2013): 1211-8.
7. Stopsack, K. H., T. A. Gerke, J. A. Sinnott, K. L. Penney, S. Tyekucheva, H. D. Sesso, S. O. Andersson, O. Andrén, J. R. Cerhan, E. L. Giovannucci, L. A. Mucci, and J. R. Rider.

"Cholesterol Metabolism and Prostate Cancer Lethality." *Cancer Res* 76, no. 16 (2016): 4785-90.

8. Stopsack, K. H., T. A. Gerke, O. Andrén, S. O. Andersson, E. L. Giovannucci, L. A. Mucci, and J. R. Rider. "Cholesterol Uptake and Regulation in High-Grade and Lethal Prostate Cancers." *Carcinogenesis* 38, no. 8 (2017): 806-11.
9. Kalogirou, C., J. Linxweiler, P. Schmucker, M. T. Snaebjornsson, W. Schmitz, S. Wach, M. Krebs, E. Hartmann, M. Puhr, A. Müller, M. Spahn, A. K. Seitz, T. Frank, H. Marouf, G. Büchel, M. Eckstein, H. Kübler, M. Eilers, M. Saar, K. Junker, F. Röhrig, B. Kneitz, M. T. Rosenfeldt, and A. Schulze. "Mir-205-Driven Downregulation of Cholesterol Biosynthesis through Sqle-Inhibition Identifies Therapeutic Vulnerability in Aggressive Prostate Cancer." *Nat Commun* 12, no. 1 (2021): 5066.
10. Xu, Z., L. Huang, T. Dai, X. Pei, L. Xia, G. Zeng, M. Ye, K. Liu, F. Zeng, W. Han, and S. Jiang. "Sqle Mediates Metabolic Reprogramming to Promote Ln Metastasis in Castration-Resistant Prostate Cancer." *Onco Targets Ther* 14 (2021): 4285-95.
11. Alfaqih, M. A., E. R. Nelson, W. Liu, R. Safi, J. S. Jasper, E. Macias, J. Geradts, J. W. Thompson, L. G. Dubois, M. R. Freeman, C. Y. Chang, J. T. Chi, D. P. McDonnell, and S. J. Freedland. "Cyp27a1 Loss Dysregulates Cholesterol Homeostasis in Prostate Cancer." *Cancer Res* 77, no. 7 (2017): 1662-73.
12. Yue, S., J. Li, S. Y. Lee, H. J. Lee, T. Shao, B. Song, L. Cheng, T. A. Masterson, X. Liu, T. L. Ratliff, and J. X. Cheng. "Cholesteryl Ester Accumulation Induced by Pten Loss and Pi3k/Akt Activation Underlies Human Prostate Cancer Aggressiveness." *Cell Metab* 19, no. 3 (2014): 393-406.
13. de Gonzalo-Calvo, D., L. López-Vilaró, L. Nasarre, M. Perez-Olabarria, T. Vázquez, D. Escuin, L. Badimon, A. Barnadas, E. Lerma, and V. Llorente-Cortés. "Intratumor Cholesteryl Ester Accumulation Is Associated with Human Breast Cancer Proliferation and Aggressive Potential: A Molecular and Clinicopathological Study." *BMC Cancer* 15 (2015): 460.
14. Poirot, M., R. Soules, A. Mallinger, F. Dalenc, and S. Silvente-Poirot. "Chemistry, Biochemistry, Metabolic Fate and Mechanism of Action of 6-Oxo-Cholestan-3β,5α-Diol (Ocdo), a Tumor Promoter and Cholesterol Metabolite." *Biochimie* 153 (2018): 139-49.

15. Wang, B., and P. Tontonoz. "Liver X Receptors in Lipid Signalling and Membrane Homeostasis." *Nat Rev Endocrinol* 14, no. 8 (2018): 452-63.
16. Bovenga, F., C. Sabbà, and A. Moschetta. "Uncoupling Nuclear Receptor Lxr and Cholesterol Metabolism in Cancer." *Cell Metab* 21, no. 4 (2015): 517-26.
17. Pehkonen, P., L. Welter-Stahl, J. Diwo, J. Ryynänen, A. Wienecke-Baldacchino, S. Heikkinen, E. Treuter, K. R. Steffensen, and C. Carlberg. "Genome-Wide Landscape of Liver X Receptor Chromatin Binding and Gene Regulation in Human Macrophages." *BMC Genomics* 13 (2012): 50.
18. Allott, E. H., E. M. Ebot, K. H. Stopsack, A. G. Gonzalez-Feliciano, S. C. Markt, K. M. Wilson, T. U. Ahearn, T. A. Gerke, M. K. Downer, J. R. Rider, S. J. Freedland, T. L. Lotan, P. W. Kantoff, E. A. Platz, M. Loda, M. J. Stampfer, E. Giovannucci, C. J. Sweeney, S. P. Finn, and L. A. Mucci. "Statin Use Is Associated with Lower Risk of Pten-Null and Lethal Prostate Cancer." *Clin Cancer Res* 26, no. 5 (2020): 1086-93.
19. Alfaqih, M. A., E. H. Allott, R. J. Hamilton, M. R. Freeman, and S. J. Freedland. "The Current Evidence on Statin Use and Prostate Cancer Prevention: Are We There Yet?" *Nat Rev Urol* 14, no. 2 (2017): 107-19.
20. Bjarnadottir, O., Q. Romero, P. O. Bendahl, K. Jirström, L. Rydén, N. Loman, M. Uhlén, H. Johannesson, C. Rose, D. Grabau, and S. Borgquist. "Targeting Hmg-Coa Reductase with Statins in a Window-of-Opportunity Breast Cancer Trial." *Breast Cancer Res Treat* 138, no. 2 (2013): 499-508.
21. Wang, Y., S. You, S. Su, A. Yeon, E. M. Lo, S. Kim, J. L. Mohler, M. R. Freeman, and H. L. Kim. "Cholesterol-Lowering Intervention Decreases Mtor Complex 2 Signaling and Enhances Antitumor Immunity." *Clin Cancer Res* 28, no. 2 (2022): 414-24.
22. Jetten, A. M., and D. N. Cook. "(Inverse) Agonists of Retinoic Acid-Related Orphan Receptor Γ: Regulation of Immune Responses, Inflammation, and Autoimmune Disease." *Annu Rev Pharmacol Toxicol* 60 (2020): 371-90.
23. Pandya, V. B., S. Kumar, Sachchidanand, R. Sharma, and R. C. Desai. "Combating Autoimmune Diseases with Retinoic Acid Receptor-Related Orphan Receptor-Γ (Rorγ or Rorc) Inhibitors: Hits and Misses." *J Med Chem* 61, no. 24 (2018): 10976-95.
24. Sun, N., H. Guo, and Y. Wang. "Retinoic Acid Receptor-Related Orphan Receptor Gamma-T (Rorγt) Inhibitors in Clinical Development for the Treatment of Autoimmune

Diseases: A Patent Review (2016-Present)." *Expert Opin Ther Pat* 29, no. 9 (2019): 663-74.

25. Wang, J., J. X. Zou, X. Xue, D. Cai, Y. Zhang, Z. Duan, Q. Xiang, J. C. Yang, M. C. Louie, A. D. Borowsky, A. C. Gao, C. P. Evans, K. S. Lam, J. Xu, H. J. Kung, R. M. Evans, Y. Xu, and H. W. Chen. "Ror-Γ Drives Androgen Receptor Expression and Represents a Therapeutic Target in Castration-Resistant Prostate Cancer." *Nat Med* 22, no. 5 (2016): 488-96.
26. Cai, D., J. Wang, B. Gao, J. Li, F. Wu, J. X. Zou, J. Xu, Y. Jiang, H. Zou, Z. Huang, A. D. Borowsky, R. J. Bold, P. N. Lara, J. J. Li, X. Chen, K. S. Lam, K. F. To, H. J. Kung, O. Fiehn, R. Zhao, R. M. Evans, and H. W. Chen. "Rorγ Is a Targetable Master Regulator of Cholesterol Biosynthesis in a Cancer Subtype." *Nat Commun* 10, no. 1 (2019): 4621.
27. Zhang, X., Z. Huang, J. Wang, Z. Ma, J. Yang, E. Corey, C. P. Evans, A. M. Yu, and H. W. Chen. "Targeting Feedforward Loops Formed by Nuclear Receptor Rorγ and Kinase Pbk in Mcrpc with Hyperactive Ar Signaling." *Cancers (Basel)* 13, no. 7 (2021).
28. Zhang, Y., X. Wu, X. Xue, C. Li, J. Wang, R. Wang, C. Zhang, C. Wang, Y. Shi, L. Zou, Q. Li, Z. Huang, X. Hao, K. Loomes, D. Wu, H. W. Chen, J. Xu, and Y. Xu. "Discovery and Characterization of Xy101, a Potent, Selective, and Orally Bioavailable Rorγ Inverse Agonist for Treatment of Castration-Resistant Prostate Cancer." *J Med Chem* 62, no. 9 (2019): 4716-30.
29. Chang, M. R., V. Dharmarajan, C. Doebelin, R. D. Garcia-Ordonez, S. J. Novick, D. S. Kuruvilla, T. M. Kamenecka, and P. R. Griffin. "Synthetic Rorγt Agonists Enhance Protective Immunity." *ACS Chem Biol* 11, no. 4 (2016): 1012-8.
30. Mahalingam, D., J. S. Wang, E. P. Hamilton, J. Sarantopoulos, J. Nemunaitis, G. Weems, L. Carter, X. Hu, M. Schreeder, and H. J. Wilkins. "Phase 1 Open-Label, Multicenter Study of First-in-Class Rorγ Agonist Lyc-55716 (Cintirorgon): Safety, Tolerability, and Preliminary Evidence of Antitumor Activity." *Clin Cancer Res* 25, no. 12 (2019): 3508-16.
31. Wang, X., B. Sun, L. Wei, X. Jian, K. Shan, Q. He, F. Huang, X. Ge, X. Gao, N. Feng, and Y. Q. Chen. "Cholesterol and Saturated Fatty Acids Synergistically Promote the Malignant Progression of Prostate Cancer." *Neoplasia* 24, no. 2 (2022): 86-97.

32. Kumar, R., A. L. Theiss, and K. Venuprasad. "Rorγt Protein Modifications and Il-17-Mediated Inflammation." *Trends Immunol* 42, no. 11 (2021): 1037-50.
33. Chang, M. R., H. Rosen, and P. R. Griffin. "Rors in Autoimmune Disease." *Curr Top Microbiol Immunol* 378 (2014): 171-82.
34. Zhao, W., Z. J. Xiao, and S. P. Zhao. "The Benefits and Risks of Statin Therapy in Ischemic Stroke: A Review of the Literature." *Neurol India* 67, no. 4 (2019): 983-92.
35. Bellosta, S., and A. Corsini. "Statin Drug Interactions and Related Adverse Reactions: An Update." *Expert Opin Drug Saf* 17, no. 1 (2018): 25-37.
36. Lytle, N. K., L. P. Ferguson, N. Rajbhandari, K. Gilroy, R. G. Fox, A. Deshpande, C. M. Schürch, M. Hamilton, N. Robertson, W. Lin, P. Noel, M. Wartenberg, I. Zlobec, M. Eichmann, J. A. Galván, E. Karamitopoulou, T. Gilderman, L. A. Esparza, Y. Shima, P. Spahn, R. French, N. E. Lewis, K. M. Fisch, R. Sasik, S. B. Rosenthal, M. Kritzik, D. Von Hoff, H. Han, T. Ideker, A. J. Deshpande, A. M. Lowy, P. D. Adams, and T. Reya. "A Multiscale Map of the Stem Cell State in Pancreatic Adenocarcinoma." *Cell* 177, no. 3 (2019): 572-86.e22.
37. Chen, J., Y. Hu, J. Zhang, Q. Wang, X. Wu, W. Huang, Q. Wang, G. Cai, H. Wang, T. Ou, W. Feng, P. Liu, Y. Liu, J. Wang, J. Huang, and J. Wang. "Therapeutic Targeting Rorγ with Natural Product N-Hydroxyapiosporamide for Small Cell Lung Cancer by Reprogramming Neuroendocrine Fate." *Pharmacol Res* 178 (2022): 106160.
38. Font-Díaz, J., A. Jiménez-Panizo, C. Caelles, M. D. Vivanco, P. Pérez, A. Aranda, E. Estébanez-Perpiñá, A. Castrillo, M. Ricote, and A. F. Valledor. "Nuclear Receptors: Lipid and Hormone Sensors with Essential Roles in the Control of Cancer Development." *Semin Cancer Biol* 73 (2021): 58-75.
39. Zadra, G., and M. Loda. "Metabolic Vulnerabilities of Prostate Cancer: Diagnostic and Therapeutic Opportunities." *Cold Spring Harb Perspect Med* 8, no. 10 (2018).
40. Tarantino, G., F. Crocetto, C. D. Vito, R. Martino, S. D. Pandolfo, M. Creta, A. Aveta, C. Buonerba, and C. Imbimbo. "Clinical Factors Affecting Prostate-Specific Antigen Levels in Prostate Cancer Patients Undergoing Radical Prostatectomy: A Retrospective Study." *Future Sci OA* 7, no. 3 (2021): Fso643.
41. Crocetto, F., S. D. Pandolfo, A. Aveta, R. Martino, F. Trama, V. F. Caputo, B. Barone, M. Abate, E. Sicignano, S. Cilio, G. Russo, M. Massanova, C. Di Vito, C. Imbimbo, and G.

Tarantino. "A Comparative Study of the Triglycerides/Hdl Ratio and Pseudocholinesterase Levels in Patients with Bladder Cancer." *Diagnostics (Basel)* 12, no. 2 (2022).

42. Hryniewicz-Jankowska, A., K. Augoff, and A. F. Sikorski. "The Role of Cholesterol and Cholesterol-Driven Membrane Raft Domains in Prostate Cancer." *Exp Biol Med (Maywood)* 244, no. 13 (2019): 1053-61.
43. Zhuang, L., J. Kim, R. M. Adam, K. R. Solomon, and M. R. Freeman. "Cholesterol Targeting Alters Lipid Raft Composition and Cell Survival in Prostate Cancer Cells and Xenografts." *J Clin Invest* 115, no. 4 (2005): 959-68.
44. Li, Y. C., M. J. Park, S. K. Ye, C. W. Kim, and Y. N. Kim. "Elevated Levels of Cholesterol-Rich Lipid Rafts in Cancer Cells Are Correlated with Apoptosis Sensitivity Induced by Cholesterol-Depleting Agents." *Am J Pathol* 168, no. 4 (2006): 1107-18; quiz 404-5.
45. Stuchbery, R., P. J. McCoy, C. M. Hovens, and N. M. Corcoran. "Androgen Synthesis in Prostate Cancer: Do All Roads Lead to Rome?" *Nat Rev Urol* 14, no. 1 (2017): 49-58.
46. Sharifi, N., and R. J. Auchus. "Steroid Biosynthesis and Prostate Cancer." *Steroids* 77, no. 7 (2012): 719-26.
47. Santori, F. R., P. Huang, S. A. van de Pavert, E. F. Douglass, Jr., D. J. Leaver, B. A. Haubrich, R. Keber, G. Lorbek, T. Konijn, B. N. Rosales, D. Rozman, S. Horvat, A. Rahier, R. E. Mebius, F. Rastinejad, W. D. Nes, and D. R. Littman. "Identification of Natural Rorgamma Ligands That Regulate the Development of Lymphoid Cells." *Cell Metab* 21, no. 2 (2015): 286-98.
48. Jin, L., D. Martynowski, S. Zheng, T. Wada, W. Xie, and Y. Li. "Structural Basis for Hydroxycholesterols as Natural Ligands of Orphan Nuclear Receptor Rorgamma." *Mol Endocrinol* 24, no. 5 (2010): 923-9.
49. Soroosh, P., J. Wu, X. Xue, J. Song, S. W. Sutton, M. Sablad, J. Yu, M. I. Nelen, X. Liu, G. Castro, R. Luna, S. Crawford, H. Banie, R. A. Dandridge, X. Deng, A. Bittner, C. Kuei, M. Tootoonchi, N. Rozenkrants, K. Herman, J. Gao, X. V. Yang, K. Sachen, K. Ngo, W. P. Fung-Leung, S. Nguyen, A. de Leon-Tabaldo, J. Blevitt, Y. Zhang, M. D. Cummings, T. Rao, N. S. Mani, C. Liu, M. McKinnon, M. E. Milla, A. M. Fourie, and S. Sun. "Oxysterols Are Agonist Ligands of Rorγt and Drive Th17 Cell Differentiation." *Proc Natl Acad Sci U S A* 111, no. 33 (2014): 12163-8.

Chapter 2

Functions of histone deacetylases in treatment-induced neuroendocrine prostate cancer

2.1 Abstract

Treatment-induced neuroendocrine prostate cancer (tNEPC) is an advanced, aggressive, and treatment-resistant subtype of prostate cancer that has lost dependency on androgen receptor (AR) signaling and develops neuroendocrine traits. However, the mechanisms by which tNEPC cells survive and proliferate are still poorly understood. Histone deacetylases (HDACs) have been studied in various types of cancers, and a number of HDAC inhibitors has been developed and tested in clinical studies. In this study, we demonstrate that in tNEPC cells, HDACs play vital role in tNEPC cell survival and proliferation, while also possess potential anti-cancer functions. First, we found that HDAC inhibition blocks NEPC cell growth. Notably, HDAC pan-inhibitors and some specific HDAC-selective inhibitors display high potency in tNEPC cell growth inhibition. Next, we found that HDAC inhibitors downregulate cell cycle and cell survival related gene programs. Interestingly, some pro-metastasis and neuroendocrine development gene programs are induced by HDAC inhibitors. Finally, using specific HDAC-selective inhibitors, we identified several potential candidates that play essential roles in tNEPC cells, including HDAC1, HDAC3, HDAC4, and HDAC5. Together, our findings identify HDACs as potential therapeutic targets for tNEPC and point out that additional target-specific compounds with less non-intended side-effects need to be developed.

2.2 Introduction

Nerves are predominantly known for their transmission of signals from the central nervous system to tissues and organs. They also play important roles during tissue development and regeneration[1,2]. During embryogenesis, nervous system development in the tissue is necessary for growth and morphogenesis, while denervation prevents organogenesis, hematopoiesis, and angiogenesis[1, 3, 4]. Neurogenesis involves the de novo production of functional neurons from neural precursors, and innervation is required for tissue regeneration following the injury[5, 6]. In cancer, newly formed nerve fibers infiltrate in various solid tumor types, including prostate, breast, pancreatic, and gastric cancers, where cancer cells surround nerve cells, leading to perineural invasion[7-11]. Axonal outgrowth from pre-existing nerves is promoted by neurotrophic factors from the tumor microenvironment, and develop into tumor-associated nervous systems that transmit neural signaling for the regulation of tumorigenesis and metastasis, which was first demonstrated in prostate cancer[8, 11, 12].

Prostate cancer, an androgen driven disease, is the most common malignancy and second leading cause of cancer-related death of men in the US[13]. The common treatment for prostate cancer is androgen deprivation therapy (ADT) and androgen receptor (AR) antagonists that target AR signaling, but at later stages, resistance to AR signaling inhibition usually occurs, and the cancer develop into a more aggressive castration resistant prostate cancer (CRPC) or anti-AR drug resistance disease[14]. Despite the resistance to ADT, CRPC are still dependent on AR signaling through mutation, amplification, or re-activation of AR[15-17]. However, a small portion of CRPC tumors lose dependence on AR signaling during the course of disease and transform into poorly differentiated small cell neuroendocrine carcinoma, which is referred to as treatment-induced neuroendocrine prostate cancer (tNEPC)[18]. The tNEPC tumors usually feature low AR

expression and are less dependent on AR signaling, but the disease is highly aggressive and metastatic.

Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from the lysine residues of both histone and non-histone proteins. There are 18 human HDACs grouped into 4 classes. Class I includes HDAC1, 2, 3, 8; class IIA includes HDAC4, 5, 7, 9; class IIB includes HDAC6, 10; and class IV includes HDAC11. HDACs play crucial roles in modulation of chromatin structure and gene expressions [19]. Abnormal functions or alterations of expression in HDACs are tightly associated with cancer development. Aberrant expression of HDACs is associated to multiple types of cancers and poor prognosis, including prostate carcinomas, ER-positive and triple negative breast cancers, pancreatic ductal adenocarcinoma, hepatocellular carcinoma, non-small cell lung cancer, and various types of hematologic cancers[19-22]. Mutations and low expression of HDACs are also found to promote several cancer types, including lung cancer, gastric cancer, and hepatocellular carcinoma[23-26]. Deregulation of HDACs promote cancer development by deacetylating histone and nonhistone proteins that are involved in the regulation of cell cycle, apoptosis, DNA damage response, angiogenesis, metastasis, and autophagy[27-35].

Since HDACs have been implicated in multiple types of cancers, a large number of HDAC inhibitors have been developed for cancer studies and treatments. These compounds modulate the activities of HDACs in their ability to modify histone and nonhistone proteins, thereby inhibit cancer cell proliferation and sensitize cancer cells to chemotherapy[36]. The Food and Drug Administration (FDA) has approved several HDAC inhibitors, including vorinostat, romidepsin, belinostat, and panobinostat, for certain types of cancers such as T-cell lymphoma and multiple myeloma[37, 38]. However, the mechanisms of cancer suppression by HDAC inhibitors varies

with different cancer types, and many of the HDAC inhibitors have side effects, including ventricular tachyarrhythmia, anemia, hypoxia, and hyperglycemia. Thus, more comprehensive studies need to be done to better understand how HDAC functions in different types of cancer cells and to develop novel, more specific HDAC inhibitors with less side effects[39-41].

In prostate cancer, the overexpression of HDAC1-4 has been associated with disease severity and poor prognosis in patients[42-45]. Thus, clinical trials attempted to use several HDAC inhibitors on CRPC patients based on their success in hematologic cancers, but there has not been any success due to either toxicity or disease progression[46]. One possible explanation to such failures is that these HDAC inhibitors possess ability to induce epithelial to mesenchymal transition (EMT) and neuroendocrine signaling, thus promote disease progression and facilitate the transition to a more aggressive disease[46-48]. In this study, we used several pan- and type-specific HDAC inhibitors and analyzed their effects on NEPC cell survival and proliferation. We further analyzed the changes in gene expression profile of NEPC cells caused by HDAC inhibitors. Our findings in NEPC cells are in accordance with the previous studies done in CRPC. We also identify HDACs as potential therapeutic targets for NEPC and demonstrate the capability of HDAC inhibitors as potential NEPC therapeutics.

2.3 Materials and Methods

Cell Culture

42D treatment-induced neuroendocrine prostate cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Gemini or Hyclone) and 10 μM Enzalutamide. Cells were grown at 37 °C in 5% CO2 incubators. 42D cell line was obtained from Dr. Amina Zoubeidi's Laboratory in Vancouver Prostate Centre, Vancouver, British Columbia, Canada[51].

Chemicals

MK0683, LBH589, CUDC101, PCI-24781, MS-275, CAY10683, RGFP966, and LMK-235 were obtained from Sigma-Aldrich, Selleck, or MedChemExpress.

Cell Viability and Growth Assays

For cell viability, cells were seeded in 96-well plates at 2000 cells per well in a total of 100 µl of media supplemented with 10 µM of Enzalutamide. Serially diluted small molecule compounds in 100 µl of media were added to each designated well after 24h. After 4 days of treatment, culture media was removed and 50 µl of Cell-Titer GLO reagents (Promega) was added, and then luminescence was measured on Varioskan Lux multimode microplate reader (ThermoFisher Scientific). All experimental points were measured as triplicates, and the experiments were repeated at least two or three times. The luminescence of cells treated with vehicle was set at 100% viability, and all other data were standardized to percentage of viable cells.

RNA-seq and Data Analysis

42D cells were treated with vehicle, MK0683 (2.5 or 5 µM), MS-275 (0.5 or 1 µM), CAY10683 (1.25 or 2.5 µM), RGFP966 (5 or 10 µM), or LMK-235 (0.25 or 0.5 µM) for 48h before RNA extraction. RNA-seq libraries from 1 µg of total RNA were prepared using Illumina Tru-Seq RNA Sample Prep Kit according to the manufacturer's instructions. Libraries were validated with Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Sequencing was performed by Illumina HiSeq 2000 sequencer at BGI Tech (Hong Kong). The FASTQ-formatted sequence data were analyzed by using a standard BWA-Bowtie-Cufflinks workflow. Sequence reads were mapped to human-genome assembly (GRCh37/hg19) with BWA and Biotite software. Cufflinks package was utilized for transcript assembly, quantification of normalized gene and isoform

expression in RPKM (reads per kilobase per million mapped reads) or FPKM (Fragments per kilobase of exon model per million mapped reads), and analysis of differential expression.

Statistical Analysis

All statistical details are included in the figure legends or the specific Method sections. The data are presented as mean values ± s.d. Statistical analysis was performed using two tailed Student's t tests to compare the means. $P < 0.05$ was considered to be statistically significant.

2.4 Results

2.4.1 HDAC inhibitors inhibit NEPC and CRPC cell growth and survival

To investigate the functions of HDACs on tNEPC cell proliferation and survival, we first treated 42D cells with two HDAC pan inhibitors and two HDAC Class I and II inhibitors. We found that all four HDAC inhibitors are very potent in inhibiting 42D cell growth, indicating that the HDACs play vital roles in 42D cell survival (Fig. 2.1 a-d). To further study the importance of specific HDACs, we treated 42D cells with more target-specific HDAC inhibitors. HDAC1, 2, 3 inhibitor MS-275 showed very high potency in growth inhibition similar to HDAC pan inhibitors (Fig. 2.1e). HDAC3 specific inhibitor RGFP966 had much less potency in growth inhibition (Fig. 2.1f), while HDAC2 specific inhibitor CAY10683 did not show any growth inhibition effect (Fig. 2.1g). Together, these data indicate that both HDAC1 and HDAC3 play important roles in 42D cell growth and survival. In addition, HDAC4 and 5 inhibitor LMK-235 also showed similar potency in growth inhibition, indicating that HDAC4 or HDAC5 may also be necessary for 42D cell survival. (Fig. 2.1h). To compare HDAC functions on cell survival in CRPC, we also treated C4-2B cells with the same panel of HDAC inhibitors. The results were very similar to the results from 42D cells (Fig. 2.1i-p). Notably, C4-2B cells were more responsive to HDAC3 and HDAC4, 5

inhibitors, suggesting that 42D cell may possess stronger abilities to utilize different HDACs to compensate the loss of functions of several other HDACs.

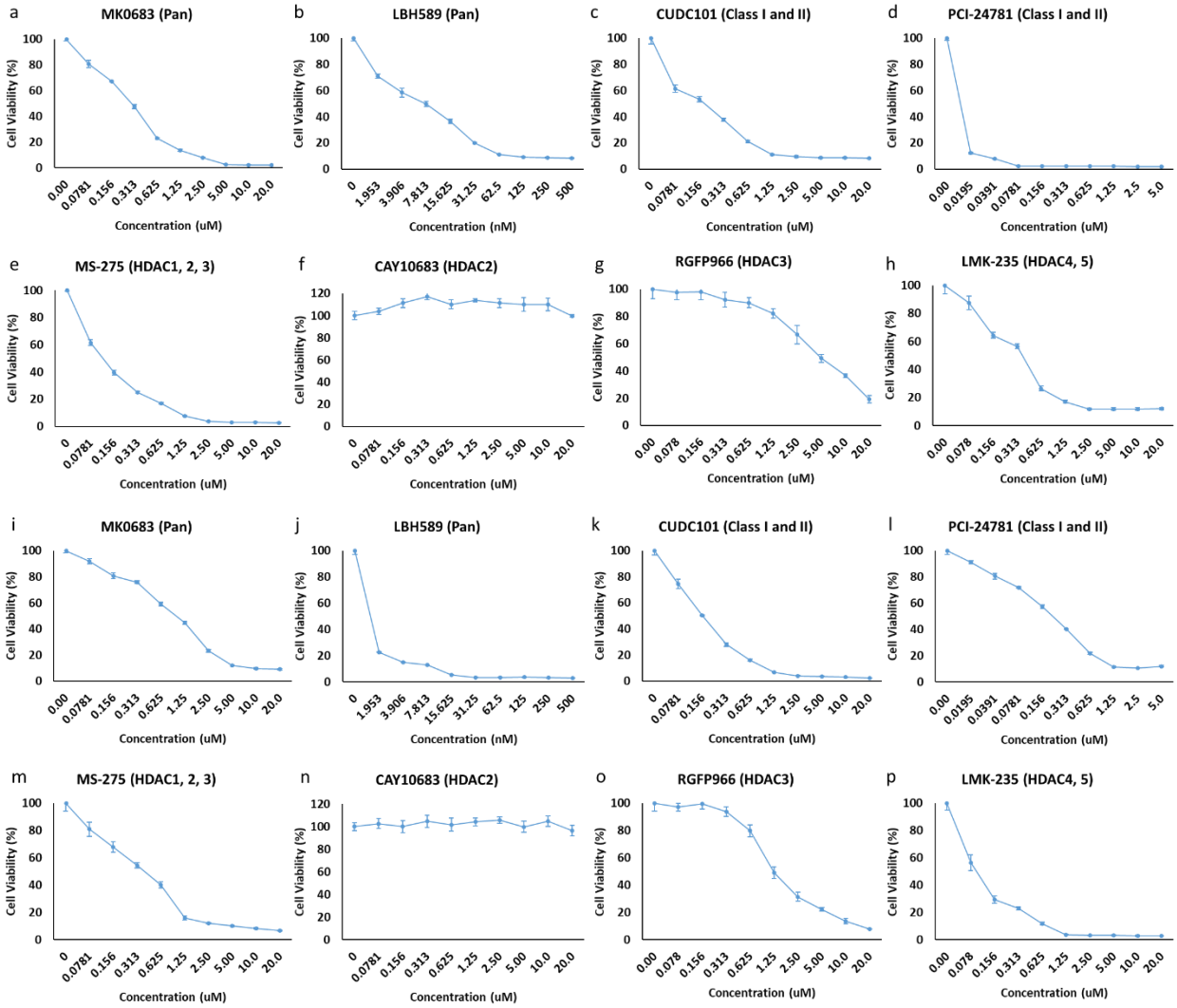


Figure 2.1 HDAC inhibitors inhibit NEPC and CRPC cell growth and survival. (**a-h**) Cell Viability, measured by Cell-Titer GLO (Promega) of 42D cells treated with the indicated concentration of HDAC inhibitors MK0683, LBH589, CUDC101, PCI-24781, MS-275, CAY10683, RFGP966, and LMK-235 for 4d. (**i-p**) C4-2B cells treated with the indicated concentration of HDAC inhibitors MK0683, LBH589, CUDC101, PCI-24781, MS-275, CAY10683, RFGP966, and LMK-235 for 4 d. Data are shown as mean ± s.d. n = 3. Student's t test. $*p < 0.05$, $**p < 0.01$.

2.4.2 HDAC pan inhibitor MK0683 downregulates cell survival and replication gene programs and upregulates neuroendocrine signaling pathways

HDACs modify both histone and non-histone proteins, thus playing crucial roles in a wide range of gene expressions. To acquire a more comprehensive understanding on HDAC functions, we treated 42D cells with HDAC pan inhibitor MK0683 and performed RNA-seq analysis. We then gathered the most downregulated and upregulated genes and used these two clusters of genes to perform gene ontology analysis. Our analysis showed that translation, cell division, DNA repair, and cell cycle are among the most downregulated gene programs by MK0683 treatment in 42D cells (Fig. 2.2a). Accordant with the cell viability assay, MK0683 treatment largely impaired the cell proliferation and survival related gene programs. On the other hand, nervous system development, neuron differentiation, angiogenesis, and positive regulation of cell migration are among the most upregulated gene programs by MK0683 (Fig. 2.2b). Surprisingly, despite its effect on growth inhibition, HDAC pan inhibition stimulated pro-neuroendocrine differentiation and pro-metastasis gene programs. To further demonstrate the effect of HDAC pan inhibition on the expression of neuroendocrine signaling pathways, we analyzed the changes in expression of key neuroendocrine markers and drivers (Fig. 2.2c). Strikingly, most of the key neuroendocrine driver and marker genes are significantly induced, including POU3F2 (BRN2), FOXA1, SOX2, and SYP. One exception is ASCL1, which expression is slightly downregulated by the treatment.

a

5 µM MK0683 DOWN	
Description	LogP
cytoplasmic translation	-44.00
translation	-34.72
cell division	-10.07
DNA replication	-9.59
mitochondrial translation	-9.11
mitochondrial ATP synthesis coupled proton transport	-7.18
DNA repair	-5.19
mitotic cell cycle	-4.10

b

5 µM MK0683 UP	
Description	LogP
cell adhesion	-24.82
nervous system development	-20.07
axon guidance	-9.02
chemical synaptic transmission	-8.30
synapse assembly	-6.82
angiogenesis	-6.74
positive regulation of cell migration	-5.35
neuron differentiation	-4.82

c

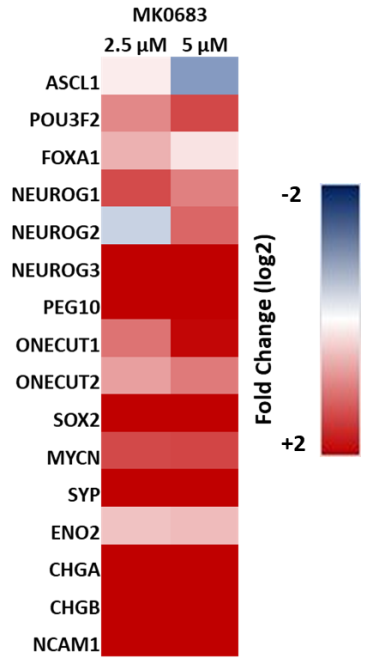


Figure 2.2 HDAC pan inhibitor MK0683 downregulates cell survival and replication gene programs and upregulates neuroendocrine signaling pathways. (**a**) Gene ontology analysis of the 3526 most significantly downregulated (< 0.667 fold changes) genes of 42D cells in response to 5 µM MK0683 treatment for 48h. (**b**) Gene ontology analysis of the 5879 most significantly upregulated (> 1.783 fold changes) genes of 42D cells in response to 5 µM MK0683 treatment for 48h. (**c**) Heat map of mRNA expression changes of 16 neuroendocrine driver and marker genes, as detected by RNA-seq in 42D cells treated with 2.5 µM and 5 µM of MK0683 for 48 h, as compared to vehicle (DMSO).

2.4.3 HDAC1-3 inhibitor MS-275 and HDAC3 inhibitor RGFP966 cause changes in gene expression similar to MK0683 while HDAC2 inhibitor CAY10683 is not active

Since we analyzed the effect of HDAC pan inhibitor on the changes of gene expression in 42D cells, to further acquire more detailed understanding of the importance and differences in each class I HDAC, we treated 42D cells with HDAC1-3 inhibitor MS-275, HDAC2 inhibitor CAY10683, and HDAC3 inhibitor RGFP966 and performed RNA-seq analysis. We then gathered the most downregulated and upregulated genes in each of the treatment group and used these data

to perform gene ontology analysis. Firstly, in HDAC1-3 inhibitor MS-275 treatment, translation, cell division, DNA repair, and cell cycle are among the most downregulated gene programs in 42D cells (Fig. 2.3a). The effect of MS-275 treatment was very similar to MK0683, which also impaired the cell proliferation and survival related gene programs. On the other hand, nervous system development, neuron differentiation, angiogenesis, and synapse assembly are among the most upregulated gene programs by MS-275 (Fig. 2.3b). Similar to HDAC pan inhibition, HDAC1-3 inhibition stimulated pro-neuroendocrine differentiation and angiogenesis gene programs, indicating that HDAC1, 2, and/or 3 may play important roles in cancer and metastasis suppression. Analysis of expression changes in the key neuroendocrine markers and drivers also showed very similar pattern compared to MK0863 treatment (Fig. 2.3c). Notably, ASCL1 expression was also slightly upregulated by MS-275 treatment in 42D cells. Next, we performed both gene ontology and expression change analysis on the RNA-seq data acquired from 42D cells treated with HDAC2 inhibitor CAY10683. None of the cell proliferation and survival related gene programs were found in the most downregulated genes (Fig 2.3d), which is consistent with the cell viability assay. Also, none of the pro-neuroendocrine differentiation, pro-cancer, or pro-metastasis gene programs were found in the upregulated genes (Fig 2.3e), indicating that HDAC2 may not play very important roles in cancer development in 42D cells. Expression analysis in the key neuroendocrine markers and drivers also showed no significant changes in most of the analyzed genes (Fig. 2.3f). Finally, we treated 42D cells with HDAC3 inhibitor RGFP966, and performed both gene ontology and gene expression change analysis. Based on the efficacy of RGFP966 on growth inhibition, a higher dose of treatment was performed to maximize its effect on gene expression changes. Similar to the HDAC1-3 inhibitor treatment, translation, cell division, DNA repair, and cell cycle are among the most downregulated gene programs in 42D cells, (Fig. 2.3g), indicating that HDAC3 plays vital

roles in cell proliferation and survival. Among the most downregulated genes, nervous system development, synapse assembly, and cell differentiation were the most significantly affected gene programs (Fig. 2.3h). These results disagree with a previous study, which stated that HDAC3 inhibition with RGFP966 did not cause the induction of epithelial to mesenchymal (EMT) and neuroendocrine differentiation[49]. Analysis of expression changes in the key neuroendocrine markers and drivers also showed significant induction of some key genes, including NEUROG3, SYP, CHGA, and CHGB (Fig. 2.3i).

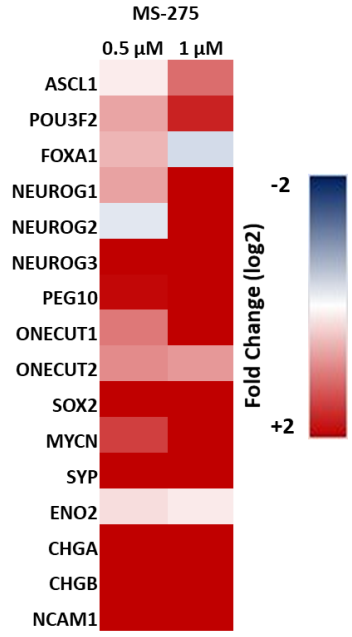
a

1 µM MS-275 DOWN	
Description	LogP
cell division	-21.85
cytoplasmic translation	-16.60
DNA replication	-15.02
translation	-12.06
mitotic cell cycle	-10.00
cell cycle	-9.30
DNA repair	-8.96
interstrand cross-link repair	-5.19

b

1 µM MS-275 UP	
Description	LogP
cell adhesion	-24.92
nervous system development	-17.96
axon guidance	-12.41
chemical synaptic transmission	-9.96
regulation of ion transmembrane transport	-7.96
synapse assembly	-7.49
angiogenesis	-6.89
neuron differentiation	-6.66

c



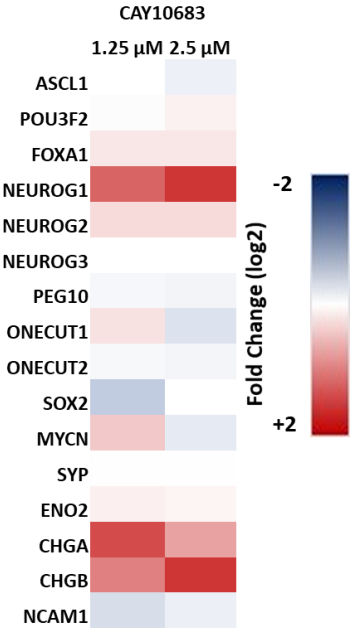
d

2.5 µM CAY10683 DOWN	
Description	LogP
cell adhesion	-7.62
ossification	-3.49
chemotaxis	-2.66
positive regulation of NIK/NF-kappaB signaling	-2.35
leukocyte migration	-2.33
signal transduction	-2.24
nervous system development	-2.13
inflammatory response	-1.92

e

2.5 µM CAY10683 UP	
Description	LogP
cell adhesion	-10.34
cell-cell signaling	-6.21
activation of adenylate cyclase activity	-4.85
anatomical structure morphogenesis	-4.62
cation transmembrane transport	-3.68
inflammatory response	-3.14
amino acid transmembrane transport	-2.82
chemical synaptic transmission	-2.42

f



g

10 µM RFGP966 DOWN	
Description	LogP
cell division	-48.10
DNA repair	-23.64
DNA replication	-21.66
mitotic cell cycle	-20.47
cell cycle	-12.96
interstrand cross-link repair	-11.10
double-strand break repair via homologous recombination	-10.14
double-strand break repair	-6.26

h

10 µM RFGP966 UP	
Description	LogP
cell adhesion	-17.23
nervous system development	-8.34
synapse assembly	-5.05
regulation of ion transmembrane transport	-4.89
cell differentiation	-4.77
modulation of synaptic transmission	-4.25
chemical synaptic transmission	-4.00
axon guidance	-3.41

i

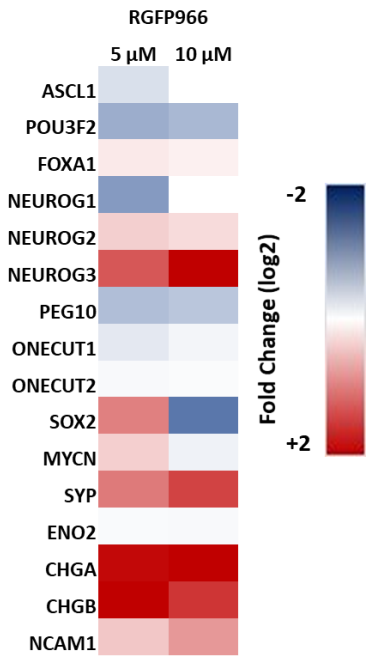


Figure 2.3 HDAC1-3 inhibitor MS-275 and HDAC3 inhibitor RGFP966 cause changes in gene expression similar to MK0683 while HDAC2 inhibitor CAY10683 is not active. (**a**) Gene ontology analysis of the 3617 most significantly downregulated (< 0.667 fold changes) genes of 42D cells in response to 1 µM MS-275 treatment for 48h. (**b**) Gene ontology analysis of the 5612 most significantly upregulated (> 1.783 fold changes) genes of 42D cells in response to 1 µM MS-275 treatment for 48h. (**c**) Heat map of mRNA expression changes of 16 neuroendocrine driver and marker genes, as detected by RNA-seq in 42D cells treated with 0.5 µM and 1 µM of MK-275 for 48 h, as compared to vehicle (DMSO). (**d**) Gene ontology analysis of the 1254 most significantly downregulated (< 0.667 fold changes) genes of 42D cells in response to 2.5 µM CAY10683 treatment for 48h. (**e**) Gene ontology analysis of the 1149 most significantly upregulated (> 1.783 fold changes) genes of 42D cells in response to 2.5 µM CAY10683 treatment for 48h. (**f**) Heat map of mRNA expression changes of 16 neuroendocrine driver and marker genes, as detected by RNA-seq in 42D cells treated with 1.25 µM and 2.5 µM of CAY10683 for 48 h, as compared to vehicle (DMSO). (**g**) Gene ontology analysis of the 2134 most significantly downregulated (< 0.667 fold changes) genes of 42D cells in response to 10 µM RGFP966 treatment for 48h. (**h**) Gene ontology analysis of the 2157 most significantly upregulated (> 1.783 fold changes) genes of 42D cells in response to 10 µM RGFP966 treatment for 48h. (**i**) Heat map

of mRNA expression changes of 16 neuroendocrine driver and marker genes, as detected by RNA-seq in 42D cells treated with 5 μM and 10 μM of RGFP966 for 48 h, as compared to vehicle (DMSO).

2.4.4 HDAC4/5 inhibitor LMK-235 downregulates cell survival and replication gene programs and upregulates neuroendocrine signaling pathways

In addition to HDAC pan inhibitors and HDAC class I specific inhibitors, HDAC4/5 inhibitor LMK-235 also exhibited significant effects in 42D cell growth inhibition. To investigate the functions and importance of HDAC4 and HDAC5, we treated 42D cells with LMK-235 and performed RNA-seq analysis. We then gathered the most downregulated and upregulated genes and used these two clusters of genes to perform gene ontology analysis. Similar to HDAC pan inhibitor and class I specific inhibitors, analysis showed that translation, DNA replication, cell division, and DNA repair, are among the most downregulated gene programs (Fig. 2.4a), and that nervous system development, synapse assembly, and angiogenesis are among the most upregulated gene programs (Fig. 2.4b). Notably, inflammatory response gene program was also significantly upregulated, which could be another pro-cancer development factor[50]. To further demonstrate the effect of HDAC4/5 inhibition on the expression of neuroendocrine signaling pathways, we analyzed the changes in expression of key neuroendocrine markers and drivers (Fig. 2.4c). Similar to HDAC pan inhibition, many neuroendocrine driver and marker genes are highly overexpressed, but some genes are only slightly upregulated, including POU3F2 (BRN2), FOXA1, and NEUROG1. The slight differences in expression changes may indicate that HDACs can compensate the loss of function for other HDACs in 42D cells.

a

0.5 µM LMK-235 DOWN	
Description	LogP
cytoplasmic translation	-52.89
translation	-40.49
mitochondrial translation	-9.41
DNA replication	-8.82
DNA repair	-5.31
chromosome segregation	-4.03
ribosome biogenesis	-3.80
cell division	-3.33

b

0.5 µM LMK-235 UP	
Description	LogP
cell adhesion	-31.21
nervous system development	-15.27
positive regulation of synapse assembly	-10.96
chemical synaptic transmission	-10.03
axon guidance	-9.82
synapse assembly	-7.18
inflammatory response	-6.10
angiogenesis	-6.05

c

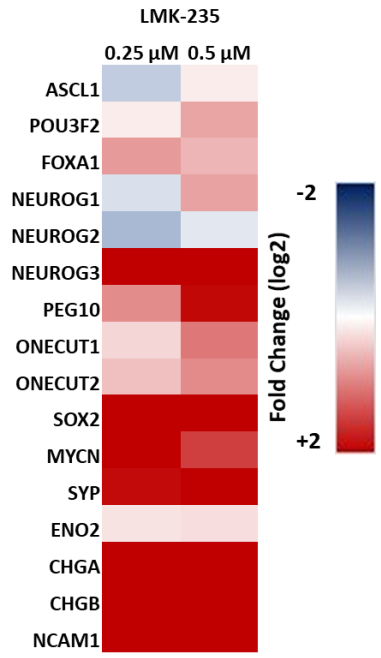


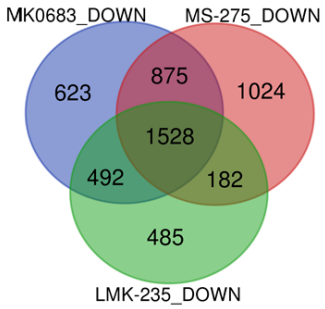
Figure 2.4 HDAC4/5 inhibitor LMK-235 downregulates cell survival and replication gene programs and upregulates neuroendocrine signaling pathways. (**a**) Gene ontology analysis of the 2693 most significantly downregulated (< 0.667 fold changes) genes of 42D cells in response to 0.5 µM LMK-235 treatment for 48h. (**b**) Gene ontology analysis of the 4491 most significantly upregulated (> 1.783 fold changes) genes of 42D cells in response to 0.5 µM LMK-235 treatment for 48h. (**c**) Heat map of mRNA expression changes of 16 neuroendocrine driver and marker genes, as detected by RNA-seq in 42D cells treated with 0.25 µM and 0.5 µM of LMK-235 for 48 h, as compared to vehicle (DMSO).

2.4.5 HDAC pan inhibitor, HDAC1-3 inhibitor and HDAC4/5 inhibitor share similar effects on cell survival and neuroendocrine signaling gene programs

To analyze and compare the overlaps in cellular functions of HDAC1, 3, 4 and 5, we first selected the significantly downregulated genes in 42D cells treated by HDAC pan inhibitor MK0683, HDAC1-3 inhibitor MS-275, or HDAC4/5 inhibitor LMK-235 and found a surprisingly great overlap of 1528 genes (Fig. 2.5a). Gene ontology analysis of the 1528 overlapped genes showed that translation, DNA repair, cell division, and cell cycle were among the most significantly

downregulated gene programs (Fig. 2.5b), therefore suggesting that the key functional HDACs all play very similar roles in supporting cell survival and proliferation. Next, we selected the significantly upregulated genes in 42D cells under the same treatments and found a more remarkable overlap of 3167 genes (Fig. 2.5c). Gene ontology analysis of the 3167 overlapped genes showed that nervous system development, cell differentiation, synapse assembly, angiogenesis, and cell migration were among the most significantly upregulated gene programs (Fig. 2.5d), which corresponded with each of the single treatment, suggesting the existence of the compensation mechanisms among HDACs in 42D cells.

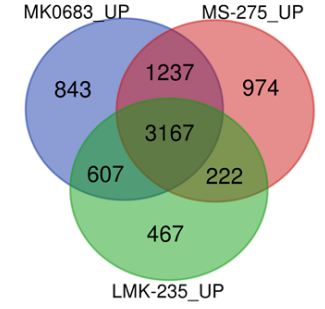
a



b

Description	LogP
cytoplasmic translation	-29.04
translation	-22.74
DNA replication	-9.92
chromosome segregation	-6.59
DNA repair	-5.15
cell division	-5.06
mitochondrial translation	-4.96
cell cycle	-4.44

c



d

Description	LogP
cell adhesion	-26.85
nervous system development	-19.40
axon guidance	-11.89
chemical synaptic transmission	-10.13
cell differentiation	-8.77
synapse assembly	-8.60
angiogenesis	-8.09
positive regulation of cell migration	-5.17

Figure 2.5 HDAC pan inhibitor, HDAC1-3 inhibitor and HDAC4/5 inhibitor share similars effect on cell survival and neuroendocrine signaling gene programs. (**a**) Venn diagram of the number of genes with expression significantly downregulated by MK0683 (5 μM), or MS-275 (1 μM), or LMK-235 (0.5 μM) in 42D cells treated for 48 h, which are detected by RNA-seq. (**b**) Gene ontology analysis of the 1528 genes overlapped in expression alterations as shown in (**a**) in response to indicated compound treatment. (**c**) Venn diagram of the number of genes with

expression significantly upregulated by MK0683 (5 μM), or MS-275 (1 μM), or LMK-235 (0.5 μM) in 42D cells treated for 48 h, which are detected by RNA-seq. (**d**) Gene ontology analysis of the 3167 genes overlapped in expression alterations as shown in (**c**) in response to indicated compound treatment.

2.5 Discussion

HDAC functions has been well investigated in various types of cancers. Since the development of various potent HDAC inhibitors, many clinical studies have been focused on utilizing them as cancer treatments. It is well known that disrupting HDAC functions is highly effective in treating hematologic cancers, leading to FDA approval of several small molecule HDAC inhibitors. However, several other clinical trials on solid tumor types are facing challenges in multiple aspects, such disease progression, adverse side effects, or patient death. This is mainly due to that the precise functions and involved mechanisms of HDAC functions in control of proliferation and tumorigenesis still remain unknown. Although HDAC inhibitors can trigger cell cycle arrest and cell death in many types of cancer, some studies also observed tumor suppressive functions of several HDACs. More studies are needed to systematically investigate the functions of each individual HDAC in different cancer types. This would require the development of particular selective HDAC inhibitors to facilitate more mechanistic and preclinical studies. Therefore, our study utilized the currently available, relatively selective HDAC inhibitors and probed individual HDAC functions in an advanced and aggressive type of prostate cancer, tNEPC.

tNEPC is an increasingly recognized subtype of prostate cancer, which arises in later stages of the disease, often from CRPC. Currently, effective therapeutics targeting neuroendocrine features of tNEPC has not yet been developed. More studies are focusing on the genomics, epigenetics, and biological features of tNEPC to acquire better understandings on the development of the cancer

and to search for potential therapeutics. Previous clinical studies utilized HDAC inhibitors on prostate cancer patients but did not succeed. Our current study may have indicated the reason for the failure of HDAC inhibitors to prevent disease progression, where HDAC inhibition causes cell cycle arrest and cell death, it also leads to upregulation of genes with functions in cancer progression and neuroendocrine signaling.

2.6 Future Directions

Our current study utilized commercially available HDAC inhibitors to facilitate our study on HDAC functions. However, due to the limitation of the current HDAC inhibitors, the specificity of the inhibitors we used was not ideal for studying the molecular functions of each individual HDAC. Our next step is to use specific siRNAs to genetically knockdown each individual HDAC and analyze the effect of the knockdown in NEPC cell lines. Given the observed possible HDAC activity compensation in the NECP cells, HDAC double or a combination of multiple knockdowns may also be performed to acquire more comprehensive understanding on the molecular functions of each HDAC and HDAC classes.

Next, based on the RNA-seq data, we acquired basic understanding on HDAC related genetic signaling pathways. However, we have found that HDAC inhibition not only caused cell cycle arrest and cell death, but also induced pro-metastasis, and neuroendocrine related signaling pathways. Since HDACs modify histone proteins to alter expression of their target genes, more genetic analysis need to be performed to identify specific HDAC targets, such as chromatin immunoprecipitation followed by sequencing (ChIP-seq) and assay for transposase-accessible chromatin using sequencing (ATAC-seq). By analyzing ChIP-seq and ATAC-seq data, we can acquire better understanding on how HDACs control cell survival and anti-cancer related signaling

pathways, and may identify potential therapeutic targets that can suppress HDAC inhibitor induced expression of pro-cancer development and neuroendocrine related genes.

Upon identification of potential therapeutics, combination treatments with HDAC inhibitors can be tested in different NEPC systems. On cell lines, RNA and protein expression of key cell survival, proliferation, cancer development, and neuroendocrine related genes can be performed. If potential combination of therapeutics can be identified, we can test whether the therapeutics can inhibit solid tumor growth using NEPC xenograft models in mice. At the same time, gene expression and the relative safety of the therapeutics can also be analyzed during the xenograft treatment, which will consolidate the combination treatment plan for potential clinical studies.

As the advances of the small molecule discovery and development, we hope to identify more potential therapeutics targeting HDACs with higher target specificity and less toxicity. Also, as the high-throughput sequencing techniques are already made available, we intend to utilize them to generate more comprehensive evidence of the beneficial effects on HDAC inhibition in advanced prostate cancer patients.

2.7 References

1. Eichmann, Anne, and Jean-Léon Thomas. "Molecular parallels between neural and vascular development." *Cold Spring Harbor perspectives in medicine* vol. 3,1 a006551. 1 Jan. 2013.
2. Kumar, Anoop, and Jeremy P Brockes. "Nerve dependence in tissue, organ, and appendage regeneration." *Trends in neurosciences* vol. 35,11 (2012): 691-9.
3. Nedvetsky, Pavel I et al. "Parasympathetic innervation regulates tubulogenesis in the developing salivary gland." *Developmental cell* vol. 30,4 (2014): 449-62.

4. Bower, Danielle V et al. “Airway branching has conserved needs for local parasympathetic innervation but not neurotransmission.” *BMC biology* vol. 12 92. 11 Nov. 2014.
5. Ming, Guo-Li, and Hongjun Song. “Adult neurogenesis in the mammalian brain: significant answers and significant questions.” *Neuron* vol. 70,4 (2011): 687-702.
6. Tanaka, Elly M, and Patrizia Ferretti. “Considering the evolution of regeneration in the central nervous system.” *Nature reviews. Neuroscience* vol. 10,10 (2009): 713-23.
7. Liebig, Catherine et al. “Perineural invasion in cancer: a review of the literature.” *Cancer* vol. 115,15 (2009): 3379-91.
8. Magnon, Claire et al. “Autonomic nerve development contributes to prostate cancer progression.” *Science (New York, N.Y.)* vol. 341,6142 (2013): 1236361.
9. Pundavela, Jay et al. “Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer.” *Molecular oncology* vol. 9,8 (2015): 1626-35.
10. Stopczynski, Rachelle E et al. “Neuroplastic changes occur early in the development of pancreatic ductal adenocarcinoma.” *Cancer research* vol. 74,6 (2014): 1718-27.
11. Hayakawa, Yoku et al. “Nerve Growth Factor Promotes Gastric Tumorigenesis through Aberrant Cholinergic Signaling.” *Cancer cell* vol. 31,1 (2017): 21-34.
12. Dobrenis, Kostantin et al. “Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development.” *International journal of cancer* vol. 136,4 (2015): 982-8.
13. Siegel, Rebecca L et al. “Cancer statistics, 2020.” *CA: a cancer journal for clinicians* vol. 70,1 (2020): 7-30.
14. Pishgar, Farhad et al. “Global, Regional and National Burden of Prostate Cancer, 1990 to 2015: Results from the Global Burden of Disease Study 2015.” *The Journal of urology* vol. 199,5 (2018): 1224-1232.
15. Beer, Tomasz M et al. “Enzalutamide in metastatic prostate cancer before chemotherapy.” *The New England journal of medicine* vol. 371,5 (2014): 424-33.
16. de Bono, Johann S et al. “Abiraterone and increased survival in metastatic prostate cancer.” *The New England journal of medicine* vol. 364,21 (2011): 1995-2005.

17. Smith, Matthew R et al. “Apalutamide Treatment and Metastasis-free Survival in Prostate Cancer.” *The New England journal of medicine* vol. 378,15 (2018): 1408-1418.

18. Abida, Wassim et al. “Genomic correlates of clinical outcome in advanced prostate cancer.” *Proceedings of the National Academy of Sciences of the United States of America* vol. 116,23 (2019): 11428-11436.

19. Seto, Edward, and Minoru Yoshida. “Erasers of histone acetylation: the histone deacetylase enzymes.” *Cold Spring Harbor perspectives in biology* vol. 6,4 a018713. 1 Apr. 2014.

20. Ramaiah, M Janaki et al. “Epigenetic modulation and understanding of HDAC inhibitors in cancer therapy.” *Life sciences* vol. 277 (2021): 119504.

21. McClure, Jesse J et al. “Advances and Challenges of HDAC Inhibitors in Cancer Therapeutics.” *Advances in cancer research* vol. 138 (2018): 183-211.

22. Yoon, Somy, and Gwang Hyeon Eom. “HDAC and HDAC Inhibitor: From Cancer to Cardiovascular Diseases.” *Chonnam medical journal* vol. 52,1 (2016): 1-11.

23. Ropero, Santiago et al. “A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition.” *Nature genetics* vol. 38,5 (2006): 566-9.

24. Osada, Hirotaka et al. “Reduced expression of class II histone deacetylase genes is associated with poor prognosis in lung cancer patients.” *International journal of cancer* vol. 112,1 (2004): 26-32.

25. Jin, Ziliang et al. “Decreased expression of histone deacetylase 10 predicts poor prognosis of gastric cancer patients.” *International journal of clinical and experimental pathology* vol. 7,9 5872-9. 15 Aug. 2014

26. Lv, Zhen et al. “Downregulation of HDAC6 promotes angiogenesis in hepatocellular carcinoma cells and predicts poor prognosis in liver transplantation patients.” *Molecular carcinogenesis* vol. 55,5 (2016): 1024-33.

27. Oehme, Ina et al. “Histone deacetylase 8 in neuroblastoma tumorigenesis.” *Clinical cancer research : an official journal of the American Association for Cancer Research* vol. 15,1 (2009): 91-9.

28. Oehme, Ina et al. “Histone deacetylase 10 promotes autophagy-mediated cell survival.” *Proceedings of the National Academy of Sciences of the United States of America* vol. 110,28 (2013): E2592-601.

29. Ecker, Jonas et al. “Targeting class I histone deacetylase 2 in MYC amplified group 3 medulloblastoma.” *Acta neuropathologica communications* vol. 3 22. 3 Apr. 2015.

30. Milde, Till et al. “HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth.” *Clinical cancer research : an official journal of the American Association for Cancer Research* vol. 16,12 (2010): 3240-52.

31. Jung, Kwang Hwa et al. “HDAC2 overexpression confers oncogenic potential to human lung cancer cells by deregulating expression of apoptosis and cell cycle proteins.” *Journal of cellular biochemistry* vol. 113,6 (2012): 2167-77.

32. Xie, Hong Jian et al. “HDAC1 inactivation induces mitotic defect and caspase-independent autophagic cell death in liver cancer.” *PloS one* vol. 7,4 (2012): e34265.

33. Buurman, Reena et al. “Histone deacetylases activate hepatocyte growth factor signaling by repressing microRNA-449 in hepatocellular carcinoma cells.” *Gastroenterology* vol. 143,3 (2012): 811-820.e15.

34. Fan, Jian et al. “Down-regulation of HDAC5 inhibits growth of human hepatocellular carcinoma by induction of apoptosis and cell cycle arrest.” *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* vol. 35,11 (2014): 11523-32.

35. Hayashi, Akiko et al. “Type-specific roles of histone deacetylase (HDAC) overexpression in ovarian carcinoma: HDAC1 enhances cell proliferation and HDAC3 stimulates cell migration with downregulation of E-cadherin.” *International journal of cancer* vol. 127,6 (2010): 1332-46.

36. West, Alison C, and Ricky W Johnstone. “New and emerging HDAC inhibitors for cancer treatment.” *The Journal of clinical investigation* vol. 124,1 (2014): 30-9.

37. Eckschlager, Tomas et al. “Histone Deacetylase Inhibitors as Anticancer Drugs.” *International journal of molecular sciences* vol. 18,7 1414. 1 Jul. 2017.

38. Marks, P A, and W-S Xu. “Histone deacetylase inhibitors: Potential in cancer therapy.” *Journal of cellular biochemistry* vol. 107,4 (2009): 600-8.

39. Ramaiah, M Janaki et al. “Epigenetic modulation and understanding of HDAC inhibitors in cancer therapy.” *Life sciences* vol. 277 (2021): 119504.

40. Ho, Terence C S et al. “Thirty Years of HDAC Inhibitors: 2020 Insight and Hindsight.” *Journal of medicinal chemistry* vol. 63,21 (2020): 12460-12484.

41. McClure, Jesse J et al. “Advances and Challenges of HDAC Inhibitors in Cancer Therapeutics.” *Advances in cancer research* vol. 138 (2018): 183-211.

42. Ropero, Santiago, and Manel Esteller. “The role of histone deacetylases (HDACs) in human cancer.” *Molecular oncology* vol. 1,1 (2007): 19-25.

43. Weichert, Wilko. “HDAC expression and clinical prognosis in human malignancies.” *Cancer letters* vol. 280,2 (2009): 168-76.

44. Weichert, W et al. “Histone deacetylases 1, 2 and 3 are highly expressed in prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical prostatectomy.” *British journal of cancer* vol. 98,3 (2008): 604-10.

45. Rhodes, Daniel R et al. “ONCOMINE: a cancer microarray database and integrated data-mining platform.” *Neoplasia (New York, N.Y.)* vol. 6,1 (2004): 1-6.

46. Rana, Zohaib et al. “Understanding Failure and Improving Treatment Using HDAC Inhibitors for Prostate Cancer.” *Biomedicines* vol. 8,2 22. 30 Jan. 2020.

47. Kong, Dejuan et al. “Histone deacetylase inhibitors induce epithelial-to-mesenchymal transition in prostate cancer cells.” *PloS one* vol. 7,9 (2012): e45045.

48. Li, Ping et al. “Contributions of epithelial-mesenchymal transition and cancer stem cells to the development of castration resistance of prostate cancer.” *Molecular cancer* vol. 13 55. 12 Mar. 2014.

49. McLeod, Abigail B et al. “Validation of histone deacetylase 3 as a therapeutic target in castration-resistant prostate cancer.” *The Prostate* vol. 78,4 (2018): 266-277.

50. Zhao, Huakan et al. “Inflammation and tumor progression: signaling pathways and targeted intervention.” *Signal transduction and targeted therapy* vol. 6,1 263. 12 Jul. 2021.

51. Bishop, Jennifer L et al. “The Master Neural Transcription Factor BRN2 Is an Androgen Receptor-Suppressed Driver of Neuroendocrine Differentiation in Prostate Cancer.” *Cancer discovery* vol. 7,1 (2017): 54-71.

Concluding Remarks

The development of small molecule therapeutics has been significantly advanced in recent years, providing us convenient and reliable tools to probe the molecular basis in the progression of advanced prostate cancer. As prostate cancer progresses, the cancer cells develop androgen deprivation treatment resistant mechanisms for their survival and proliferation. During the development of the treatment-resistance, the cancer cells differentiate into castration resistant phenotype, or sometimes acquire more aggressive and metastatic neuroendocrine traits. To develop potential therapeutics for patients suffering from these advanced types of prostate cancer, better understandings of the mechanisms by which the cancer cells acquire to achieve survivability and aggressiveness are in urgent need. Novel targeted therapeutics or combination treatments has been extensively employed and analyzed in pre-clinical and clinical studies, providing us valuable tools to conduct mechanistic studies.

Our studies focus on taking advantage in the development of the small molecule compounds and probing the mechanisms underlying the progression and aggressive of advanced prostate cancer. We have identified RORγ as a potential therapeutic target and probed its function in controlling aberrant cholesterol levels through interaction with LXRs and promote tumor growth. In addition, we have tested the potency and relative safety of small molecule compounds, either single or in a combination in treatment of preclinical models of CRPC. Also, we have studied the functions of HDACs in tNEPC cells in control of their survival and progression, and also found possible explanations for the lack of success in clinical trials of HDAC inhibitors.