A Study of the Function of RORγ in Cancer Using Synthetic and Natural Small-Molecule Modulators

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Abstract

Nuclear receptor RAR-related orphan receptor gamma (RORγ) and its isoform RORγt are major transcriptional regulators in control of mammalian metabolism, circadian rhythm, and immune responses. Recent studies have highlighted their significant roles in tumor development and progression in different cancer types, including cancers of prostate, breast, pancrease and lung. Despite being initially recognized as an orphan receptor, recent studies have unveiled many small-molecule modulators targeting RORγ/RORγt. In my dissertation studies, I investigated the cell growth-inhibitory effects of several synthetic compounds, including VTP-23, TAK828F, XY018, and GSK805 and found that they display distinct and sometimes contrasting activities in a tissue or cell-specific manner. Specifically, VTP-23 and TAK828F effectively inhibit the inflammatory gene program in Th17 cells but exhibit limited potency in inhibiting triple-negative breast cancer (TNBC) tumor cell growth. Conversely, antagonists such as XY018 and GSK805 effectively suppress tumor cell growth but modestly affect the cytokine expression in Th17 cells. My further investigations uncovered distinct chromatin accessibility alteration elicited by the RORγ modulators as a key determinant in their tissue selectivity. Similar tissue-specific activities were also observed in natural compounds targeting RORγ, such as ursolic acid (UA) and digoxin. I found that UA inhibits RORγ-dependent transactivation function, leading to the down-regulation of AR signaling in prostate cancer cells and cholesterol biosynthesis gene programs in TNBC cells. In contrast, digoxin does not affect RORγ-controlled gene programs in TNBC cells and instead up-regulates AR signaling in prostate cancer cells. These novel findings not only illustrate, for the first time, the tissue-dependent, context-specific activities of the RORγ-targeting compounds but also suggest that the mechanisms of RORγ-function in regulation of specific gene programs are tissue/cell-context dependent. Also, they will likely be valuable information for future development of RORγ-targeting compounds with context-specific activities that are tailored to the disease

treatment, including unique compounds that act as agonists in certain tissue or cells (e.g. some immune cells) and at the same time as potent antagonists in other tissue or cells (e.g. cancer cells). Such dual strategy may hold great promise for effectively treatment of different cancer types where RORγ plays a crucial role in the disease progression.

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Chapter 1

Introduction

1.1 Nuclear receptors as potential therapeutic targets

Nuclear receptors (NRs) comprise a superfamily of structurally conserved, ligand-regulated transcription factors, serving as receptors for steroid hormones and derivatives of lipids and fatty acids [1,2]. NRs share a common structure including a central DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD). Ligand binding induces a conformational change in the receptor, which results in its association with other co-regulatory proteins and regulation of gene expression. Notably, NR function is diverse and context-specific. For instance, glucocorticoid binding to glucocorticoid receptor (GR) can induce the death of thymocytes and osteoblasts or promote cell survival in liver and heart [3]. Aberrant function of NR signaling leads to proliferative, reproductive and metabolic diseases such as cancer, infertility, obesity and diabetes. Thus, the NR superfamily is one of the primary classes of therapeutic drug targets for human diseases [4,5]. For example, dexamethasone and many other potent agonists of GR are used in treatment of autoimmune and inflammatory diseases. Tamoxifen, a selective estrogen receptor modulator (SERM) acting as an antagonist in mammary tumor cells but as a partial agonist in the uterus, has been widely used for treatment of estrogen receptor (ER)-positive breast cancer [6].

1.2 RORγ and RORγt as members of the nuclear receptor super family

Similar to NRs mentioned above, recent studies have uncovered important roles played by retinoic acid receptor-related orphan receptors (RORs) in control of immunity, circadian rhythm, metabolism and certain type of cancer [7,8,9]. The RORs subfamily has three members—RORα, β and γ which are encoded respectively by RORA, RORB and RORC genes and display distinct expression patterns. RORα and RORγ are widely expressed in a variety of tissues, including kidney, lung, liver, skeletal muscle, thymus, prostate and adipose tissue.

RORβ has a restricted pattern of expression in certain regions of the central nervous system (CNS), retina and pineal gland [10,11]. Due to its critical connection to many auto-immune and inflammatory diseases, including multiple sclerosis and rheumatoid arthritis [12,13], RORγ is one of the most studied members in RORs. RORγ has two isoforms, RORγ1/RORγ and RORγ2/RORγt, which are translated from mRNAs that are transcribed from alternative promoters and have the two isoforms differ in their N-terminus. RORγ is widely expressed in liver, adipose, skeletal muscle, and kidney, while RORγt is exclusively highly expressed in thymus and other immune cells [8]. Although RORγ was initially characterized as an orphan receptor, recent studies strongly suggest that certain intermediates of cholesterol biosynthesis and specific metabolites of cholesterol and bile acids are its endogenous ligands [7,8]. Similar to GR [3], RORγ also displays tissue-specific functions. RORγ displays an oscillatory expression in liver and pancreatic β cells [9,14,15] and regulates circadian rhythm gene expression [9,16,17]. RORγ also plays a key role in gluconeogenesis, insulin sensitivity as well as lipid metabolism [18,19], whereas RORγt is critical in T helper 17 cells (Th17) differentiation, innate lymphoid cell (such as ILC3) development and functions of γδ T cells [20,21,22]. Studies from us and others demonstrated that RORγ in tumor cells can promote tumor growth and metastasis in castration-resistant prostate cancer, triple-negative breast cancer (TNBC) and pancreatic adenocarcinoma [23,24,25,26,27,28]. Thus, targeting RORγ is a promising strategy for effective treatment of autoimmune diseases and specific types of cancer.

1.3 Functions of RORγ and RORγt in metabolism, immunity and cancer

Functions of RORγ in metabolism and immunity

RORγ is involved in the control of several metabolic pathways, including gluconeogenesis, lipid metabolism and sterol metabolism [18,19,29,30]. RORγ knockout mice showed decreased hepatic gluconeogenesis, leading to improved insulin sensitivity and glucose tolerance. ChIP-

seq analysis of mouse liver tissue demonstrated that RORγ directly binds to the regulatory regions of glucose metabolism genes, including G6PC, SLC2A2, GCK, GCKR, KLF15, PPARD, PKLR and GYS2. Loss of RORγ reduced the expression of these genes in a zeitgeber time (ZT)-dependent manner [18]. Exogenous over-expression of RORγ in skeletal muscle cells increased expressions of several genes involved in lipid metabolism, such as SLC2A5, ADIPOR2, IL-15 and MSTN [19]. In addition, RORγ directly controls lipid metabolic gene INSIG2A and ELOVL3. RORγ knockout mice exhibited a reduced expression level of INSIG2A, ELOVL3 and CYP8B1, resulting in decreased cholesterol and bile acids levels in both liver and serum [29,30]. Decreased INSIG2 expression can then further activate lipogenesis through activation of SREBP1 [30].

The role of RORγt in the regulation of Th17 cell differentiation is well-studied [20]. RORγt expression can be upregulated by IL-6 and TGF-β [8,20,31]. Although not necessary for Th17 differentiation, IL-23 maintains the differentiated state by stimulating RORγt expression via inducing expression of Runx1 [32]. Induced RORγt can then bind to ROR response elements (ROREs) on various Th17 differentiation-associated gene loci, including IL-17A, IL-17F and IL-23R, activating their transcription [8]. The differentiation of regulatory T cell (Treg) or Th17 lineages also relies on the relative abundance of FOXP3 and RORγt [33,34,35]. Other than Th17 cells, RORγt also plays an important role in innate lymphoid cells. It is selectively expressed in ILC3 cells. RORγt can be regulated by Runx3 and in turn directly regulate aryl hydrocarbon receptor (AHR) gene, which is required for the development and function of ILC3 cells [36]. Moreover, RORγt-deficient mice fail to develop secondary lymphoid organs [37]. In addition, γδ T cells also express IL-17 in a RORγt-dependent manner and is involved in several autoimmune diseases [38,39].

Functions of RORγ in cancer

Research on the role of RORγ in cancer did not begin until recent years. Through a meta-analysis of NR gene expression profile in prostate cancer data sets followed by IHC analysis, a study in 2016 found that the expression level of RORγ is higher in metastatic tumors when compared with benign prostate tissue or primary prostate tumors, while RORα and RORβ appeared to have lower expression in the metastatic tumors [23]. RORγ gene knockdown and treatment with its antagonists caused castration resistant prostate cancer (CRPC) cell apoptosis and inhibited the cell proliferation and survival. RNA-seq analysis showed that AR signaling pathway is controlled by RORγ, and that AR-signaling gene expression was downregulated by RORγ antagonists. Further investigation using ChIP-seq revealed that RORγ inhibition diminished AR chromatin binding and altered genome-wide H3K27ac histone modification. Additional ChIP assays confirmed that RORγ directly binds to an RORE in the first exon region of AR gene and regulates the expression of full-length AR as well as its variant forms including AR-V7. Together these results demonstrated that RORγ plays a crucial role in control of bother AR gene overexpression and AR tumorigenic signaling in prostate cancer. Interestingly, the role of RORγ in AR expression was not observed in non-malignant, human prostate epithelial cells. Moreover, RORγ antagonists alone or combined with AR inhibitor enzalutamide significantly inhibited CRPC tumor growth and metastasis in the xenograft tumor models [23]. In addition to control of AR signaling in prostate cancer, a recent study [40] demonstrated that RORγ controls the expression of MDR1/ABCB1, which is one of the major mediators of chemotherapy drug taxane resistance in CRPC cells. It was also shown that RORγ antagonists re-sensitized taxane-resistant CRPC cells and tumors to taxanes in an AR-independent manner. Interestingly, RORγ was found to be highly elevated in doxorubicin-resistant CRPC cells to promote resistance to the chemotherapy drug [41]. Another recent study showed that RORγ can regulate genes of epithelial-mesenchymal transition (EMT) in CRPC cells [42]. PBK, one

of the EMT-related signature genes was identified as a downstream target of RORγ. RORγ positively regulates the expression of PBK which in turn stabilizes RORγ and AR proteins. Together with previous findings that RORγ activates AR gene, these recent results support a model where RORγ, PBK and AR form interlocked feedforward loops in enforcing the hyperactive signaling by AR and RORγ in CRPC [42]. Moreover, pharmacological inhibition of both RORγ and PBK synergistically inhibited the expression and function of AR and AR-V7, leading to a strong inhibition of the growth of CRPC cells and tumors [42].

Pancreatic cancer is one of the deadly cancer types due to a lack of effective therapies. RORγ was recently identified as an important player in pancreatic cancer [27]. Based on a genome-wide CRISPR screening, RORγ was reported as necessary for pancreatic cancer stem cell growth and survival. RNA-seq also showed it has a higher expression in cancer stem cells than non-stem cells. Its expression level increased dramatically in mouse tumor epithelial cells relative to normal pancreatic cells. Knockdown of RORγ led to increase in cell death, decrease in cell proliferation and depletion of stem cells. RNA-seq analysis suggested that RORγ may control gene programs of stem cell and tumorigenesis. Pharmacological inhibition of RORγ alone or combined with chemo-drug in tumor-bearing, immunocompetent mice significantly reduced tumor growth, depleted stem cell burden and improved survival. Similar effect was observed in immuno-compromised mice, indicating the anti-tumor activity of RORγ inhibitor is tumor cell-specific and Th17- and other immune cells-independent. Moreover, RORγ mRNA expression was found to positively correlate with aggressiveness of pancreatic ductal adenocarcinoma (PDAC) [27].

TNBC is an aggressive cancer subtype, largely due to its lack of effective therapeutic targets such as estrogen receptor α (ERα), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2). In a recent study, RORγ was demonstrated as an attractive therapeutic target and a master regulator of tumor cholesterol biosynthesis in TNBC [28]. First, meta-analysis of

breast cancer tumor datasets revealed that the expression of RORγ gene is significantly associated with the poor survival of TNBC patients but not with ER+ breast cancer patients. CRISPR knockout, siRNA and pharmacological inhibition of RORγ caused strong inhibition of TNBC cell growth and survival, but not on ER+ breast cancer cells and non-tumorigenic epithelial cells, further suggesting that RORγ functions selectively in a subtype of breast cancer. RNA-seq data analysis indicated that genetic and pharmacological inhibition of RORγ suppressed the mRNA and protein expression of the majority cholesterol biosynthesis genes in the TNBC cells but not in ER+ cells. In the TNBC cells, ChIP-seq analysis demonstrated that RORγ and the master regulator of cholesterol biosynthesis SREBP2 adjacently bind to promoter region of most of the cholesterol biosynthesis genes. Further studies demonstrated that RORγ interacts with SREBP2 and that the interaction between the two proteins can be diminished by the RORγ antagonist. Additional ChIP-seq analysis also demonstrated that RORγ mediates SREBP2 chromatin recruitment and activation. Treatment of tumors with RORγ inhibitors alone or in combination with cholesterol lowering drug statin led to strong tumor regression and blocking of metastasis in multiple models including patient-derived xenograft (PDX) models and an immunocompetent mouse model [28].

In summary, recent research has revealed a multi-facet role of RORγ in cancer, which include the control of tumor cholesterol biosynthesis, its regulation of key cancer drivers such as AR, major tumorigenic pathways such as the AR signaling pathway, cancer stem cell program, EMT, and therapeutic resistance.

1.4 Endogenous and synthetic ligands of RORγ

Endogenous ligands of RORγ

RORγ and its related subfamily members, RORα and RORβ, were identified initially as orphan nuclear receptors without any known physiologically relevant ligands [10]. The strong transcriptional activation activity displayed by RORγ in reporter gene assays with mammalian

cells in the absence of an exogenous ligand supported the notion that RORγ may act as a constitutive activator independent of any ligand binding-invoked regulation. In line with this notion, X ray crystallography studies showed that, without ligand binding, the LBD of RORγ adopts a transcriptionally active conformation with its C-terminal helix 12 positioned for recruiting coactivator peptides [43]. However, further structural studies revealed that the major helices of LBDs (e.g. H3, H5, H6, H7, H11 and H12) of RORs form specific pocket structures with a relatively large size that would allow high affinity interactions with small molecules with different structures. Indeed, in year 2015, RORγ as well as RORα was found to bind to specific cholesterol metabolites such as cholesterol sulfate and hydroxysterols [44]. Using in vitro binding assay and reporter gene assays performed in mammalian and insect cells, in combination with knockdown of specific cholesterol biosynthesis genes, several studies identified additional cholesterol biosynthesis intermediates (CBIs) as likely endogenous ligands of RORγ, which include zymosterol, zymosterone, 7-DHC and desmosterol whereas cholesterol, lanosterol and T-MAS displayed only weak agonist activity [44,45]. Among the oxysterols, 7β, 27-dihydroxycholesterol or 7β, 27-OHC, 7keto, 27-OHC, 27-OHC and 7α, 27-OHC (listed in the order of efficacy) appeared to possess strong activity in further activating RORγ [46]. Although several sulfated sterols such as 25-OHC sulfate, desmosterol sulfate and 5α, 6α-epoxycholestanol sulfate showed high affinity binding to RORγ LBD, only desmosterol sulfate appeared to show strong biological activities in stimulation of RORγ functions [45]. For the biological significance of the endogenous ligands identified, the studies so far have all focused on their role in modulation of the RORγt function in Th17 cell differentiation and/or the production of IL-17A. In each case, the agonistic ligands significantly enhanced the differentiation of Th17 cells. In agreement, genetic depletion of genes (e.g. MSMO1, TM7SF2, SC5D) encoding enzymes responsible for producing the different ligands strongly impaired Th17 cell development [44,45,46]. On the oxysterols, in addition to CYP27A1, a key enzyme

that produces 27-OHCs, 11β-hydroxysteroid dehydrogenase (11β-HSD) has also been shown to regulate RORγ by controlling its access to 7β, 27-OHC [46,47].

In addition to the oxysterols mentioned above, certain oxysterols in the bile acid biosynthesis pathways have also been strongly suggested as endogenous ligands of RORγ in year 2010. 7-OHCs such as 7α, 7β, or 7keto-OHC can bind to RORα and RORγ with high affinities and interestingly suppress the transactivation by RORγ [48]. 7α-OHC is the product of CYP7A1, the first and rate-limiting enzyme of bile acid biosynthesis in cholesterol metabolism. Moreover, in a screen of primary and secondary bile acids that can modulate Th17 cell differentiation, a recent study published in year 2019 showed that 3-OxoLCA can directly bind RORγ and inhibit its transcriptional activity whereas other bile acids such as 3-OxoCA and 3-OxoDCA possess much weaker activities than 3-OxoLCA [49]. Feeding a strain of mice that contain a relatively high population of Th17 cells in their small intestine with chow containing 0.3% 3-OxoLCA for a week was sufficient to decrease the Th17 cell population.

Currently, the role of CBIs, oxysterols or bile acids in control of RORγ function in other normal tissue or in cancer is largely unknown. Given that the expression and function of RORγ is likely elevated in metastatic tumors [23,28], it is conceivable that certain CBIs and oxysterols might play an important role in regulation of RORγ in tumor growth and metastasis in specific subtypes of cancer.

Ligands of RORγt identified in early years

As members of the orphan NR subfamily, the demonstration of potential functions of RORγ in the control of metabolism and immunity stirred strong interest in searching for synthetic ligands in hope of developing effective agents for treatment of inflammatory diseases and metabolic disorders. The synthetic ligands identified early on are compound T0901317 and its SR-series of derivatives[50]. The nonsteroidal benzenesulfonamide T0901317 was initially identified in year 2000 as a potent agonist of LXRs with strong activities in induction of fatty acid

biosynthesis gene program in cell culture and mice [51]. T0901317 was later shown to be somewhat promiscuous in targeting other nuclear receptors including RORα and RORγ. It binds to the RORs with good affinity and significantly inhibits their activation of G6Pase, CYP7B1 and IL-17A in reporter assays [52]. The findings that RORγt plays crucial roles in promoting CD4+ T cell differentiation to Th17 and in Th17-associated autoimmune diseases (such as psoriasis, Crohn's disease and rheumatoid arthritis) prompted the search for its synthetic ligands with therapeutic values. Through optimization of T0901317, more selective compounds were identified [11]. In year 2011, SR1001 was identified as a first-in-a-class of synthetic inverse agonist/antagonist with better selectivity to RORα and RORγ and potent activities in inhibition of Th17 cell differentiation and Th17-mediated multiple sclerosis in animal models [53]. SR1001 binds to the LBD and induces a conformational change with helix 12 re-positioning, which is linked to a switch of RORγ co-factor association from coactivator to corepressor. Through modification of the scaffold of SR1001, a study conducted in 2012 identified SR2211 as a RORγ-selective antagonist with an EC50 of 0.3 μM and more than 100-fold selectivity over RORα and LXRα in reporter gene assays [54]. SR2211 also displays potent activities in suppression of IL-17A gene expression and reduction of inflammation in animal models [54,55]. Differential hydrogen/deuterium exchange (HDX) mass spectrometry analysis demonstrated that SR2211 likely makes multiple direct contacts with the LBD of RORγ. Through structure-based optimization, many other derivatives of T0901317 with improved RORγ-inhibitory biochemical and cellular activities were also identified [56].

Other than synthetic compounds, some natural products isolated from plants or microorganisms were also identified with anti-RORγ activity in early research. Based on results from reporter gene assays, ursolic acid (UA), a pentacyclic triterpenoid presented in plants, fruits and herbs, was characterized as modulators of NRs family, specifically as agonist of PPARα [57], and antagonist of LXRα [58] and RORγt [59]. UA strongly reduces IL-17 expression in naïve

CD4+ T cells and blocks the differentiation of T helper 17 (Th17) cells. In another compound screening of over 4800 candidates using a Drosophila cell-based reporter assay system, one study conducted in 2011 identified digoxin as a potent, RORγ-selective antagonist with EC50 of close to 2 μM [60]. Digoxin can be isolated from foxglove plant. It is used in the treatment of atrial fibrillation and heart failure via inhibition of the cellular Na+/K+ ATPase. Interestingly, digoxin, not structurally related digoxigenin, can suppress IL-17A gene induction and Th17 cell differentiation. It displays a strong anti-inflammatory effect in a mouse model of Th17-mediated autoimmune disease, thus providing a prove-of-principle evidence that RORγt-targeting, small molecule therapeutics can be developed for many forms of inflammatory diseases [60].

Inverse agonists/antagonists of RORγt identified for autoimmune diseases in recent years

The excellent potency and different structural features of the few synthetic ligands identified by the early studies strongly suggest that RORγ is highly amenable to modulation by structurally distinct ligands. In the years following the pioneer work, several pharmaceutical industry and academic laboratories reported their discoveries of a large number of RORγt inverse agonists with diverse chemical structures [61,62]. The high throughput screening of a compound library was carried out either in a cell-based luciferase reporter system or in an in vitro binding assay. The reporter system was used to identify compounds that strongly suppress the transcriptional activation activity of RORγ LBD. In addition to IL-17 gene promoter-driven reporter, to facilitate the screening, the reporter could also be driven by fusion proteins of RORγ LBD and GAL4 DBD assembled at multimerized GAL4 binding sites in culture of Jurkat or other cell lines [63]. The in vitro, TR-FRET based binding screen identified compounds that could disrupt the association of recombinant RORγ LBD protein with a co-activator peptide [64,65,66]. Following the screening, hit compounds were subject to the in vitro binding assay and/or cell-based reporter assay to identify compounds with excellent profiles in binding

affinity and activation inhibition and with strong selectivity to RORγ when assayed against the other NR members. Their activity in suppression of IL-17A gene expression was also measured with CD4+ T cells. Experiments of structure-activity relationship (SAR) were usually performed to obtain candidate compounds with optimized profiles of solubility, PK and PD before they were tested in animal models of autoimmune disorders.

Structural analyses revealed that most of those ligands bind to the previously defined pocket of RORγ LBD (and thus are considered as orthosteric). Representative compounds that have entered or completed clinical trials include AUR-101 [67], RTA-1701 [68], BI-730357 [62,69] BMS-986251 [70], GSK-2981278 [71,72], GSK-805 [73], VTP-43742 [61], AZD-0284 [61], TAK-828F [74], JTE-451[75]. Most of those compounds have been at phase I or phase II clinical trials for potential use in treating autoimmune diseases. Other RORγt inhibitors such as JTE-151, ARN-6039, PF-06763809 and ABBV-157 have also been reported at clinical development for autoimmune diseases such as psoriasis [76]. Except for JTE-451, BI-730357, RTA-1701 and AUR-101, trials of the other compounds have ended after phase I or II for the autoimmune disorders largely due to their lack of expected efficacies.

In a binding-based screen followed by hit optimization for compounds that disrupt the interaction of RORγ and SRC1 coactivator, MRL-871 with indazole chemotype was identified and later found to bind to an alternative site of RORγ LBD [77]. The co-crystal structures revealed that MRL-871 binds to an allosteric pocket that is predominantly hydrophobic and formed by helices H3, H4, H11 and H12 where MRL-871 binding reorients H12 in a conformation that precludes the LBD binding by the coactivator peptide. Other biochemical and cellular assays demonstrated that MRL-871 possesses high potency and selectivity to RORγ and RORγt-dependent Th17 production of IL-17. Further SAR studies of MRL-871 led to the identification of N-(Indazol-3-yl)piperidine-4-carboxylic acid as a new lead allosteric inhibitor with attractive profiles of PK, metabolic stability, anti-Th17 differentiation potency

and potentially clean off-targeting [78]. In an in silico pharmacophore screen, a recent study identified FM26 with isoxazole chemotype as another allosteric inhibitor of RORγ [79]. Interestingly, in a competitive TR-FRET coactivator peptide recruitment assay, increasing amount of cholesterol (as an allosteric ligand) reduced the IC50 value of FM26 and thus enhanced FM26 binding to the LBD. Such a cooperative binding mode was also observed between cholesterol precursors such as desmosterol and its metabolites 20a-hydroxycholesterol and 25-hydroxycholesterol, and the other allosteric ligand MRL-871 and FM26 [80]. The discovery of a RORγ allosteric site significantly expands the strategy in searching for ligands with distinct profiles because amino acid sequences constituting the allosteric pocket are less conserved in the different RORs. Also, the existence of two pockets in one LBD would allow discovery of dual targeting drugs. In a recent prove-of-principle study, Bit-L-15 was identified as a bitopic ligand of RORγ with cholesterol and MRL-871 linked by a polyethylene glycol (PEG) linker. Bit-L-15 displayed improved potency in disrupting coactivator recruitment and improved NR selectivity over its orthosteric and allosteric parental compounds [81].

Inhibitors/antagonists of RORγ with strong anti-cancer activities

In contrast to the large number of compounds identified for inhibition of RORγt function in Th17 cell differentiation and autoimmune diseases, very few chemical structures have been reported that target RORγ in cancer cells and tumors. The finding that RORγ plays a crucial role in prostate cancer [23] prompted identification of RORγ inhibitors with high potency in cancer cells. One initial attempt was combining the structural features of SR2211 and one of the GSK compounds which displayed excellent activities in suppressing RORγt function in Th17 cells [55,73]. By combining the hexafluoropropan-2-ol group of SR2211 and the amide group of GSK805 and by structure-based optimization, the study identified XY018/compound 23 and compound 31 with amide linker to possess a potent inverse agonist with a strong activity in decreasing AR full-length and variant protein expression and AR signaling in the prostate

cancer cells [23,42,82]. Those compounds, like SR2211, displayed excellent potency in inhibition of the growth of prostate and breast cancer xenograft tumors [23,28,42,82]. Additional SAR analysis with the luciferase reporter and thermal shift assay (TSA) identified XY101 and XY123 with ethyl sulfonyl connected to a benzyl group at the side of amide linker as another potent inhibitor of RORγ with good selectivity over the other NRs and excellent activities in disruption of RORγ interaction with the coactivator peptide [83,84]. In a most recent study, a natural pentacyclic triterpenoid—Betulinic acid and its structurally optimized derivatives were shown to directly bind to RORγ in crystal structure assay. The compound 22 exhibited strong binding affinity with RORγ and potent anti-tumor efficacy in pancreatic cancer xenograft model [85]. Intriguingly, recent studies also reported that some natural products can target RORγ in different cancer cells and tumors. Elaiphylin, an antibiotic isolated from a marine species of Streptomyces, can directly bind to RORγ LBD and exert strong inhibition of its activation function including its target genes in prostate cancer cells. It also displayed strong activities in inhibition of xenograft tumor growth [24]. Another natural marine product N-hydroxyapiosporamide, also directly bound to RORγ and inhibited its transcriptional activity regulating neuroendocrine signaling in small cell lung cancer. Therapeutically, N-hydroxyapiosporamide displayed a strong anti-tumor growth effect in SCLC mice xenograft models without significant toxicity [86].

Overall, recent research highlights the diverse roles of RORγ in various metabolic pathways, immune system, and its significant impact on specific cancers, including prostate, breast, pancreatic, and triple-negative breast cancer. Many synthetic compounds and natural products exhibit potent inhibition of RORγ function, demonstrating promising outcome in both pre-clinical models and clinical trials for treating autoimmune diseases and certain cancer types. Consequently, RORγ emerges as a highly promising therapeutic target for both immune-related diseases and cancer treatment.

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Chapter 2

RORγ is a context-specific master regulator of cholesterol biosynthesis and an emerging therapeutic target in cancer and autoimmune diseases

2.1 Abstract

Aberrant cholesterol metabolism and homeostasis in the form of elevated cholesterol biosynthesis and dysregulated efflux and metabolism is well recognized as a major feature of metabolic reprogramming in solid tumors. Recent studies have emphasized on major drivers and regulators such as Myc, mutant p53, SREBP2, LXRs and oncogenic signaling pathways that play crucial roles in tumor cholesterol metabolic reprogramming. Therapeutics such as statins targeting the mevalonate pathway were tried at the clinic without showing consistent benefits to cancer patients. Nuclear receptors are prominent regulators of mammalian metabolism. Their de-regulation often drives tumorigenesis. RORγ and its immune cell-specific isoform RORγt play important functions in control of mammalian metabolism, circadian rhythm and immune responses. Although RORγ, together with its closely related members RORα and RORβ were identified initially as orphan receptors, recent studies strongly support the conclusion that specific intermediates and metabolites of cholesterol pathways serve as endogenous ligands of RORγ. More recent studies also reveal a critical role of RORγ in tumorigenesis through major oncogenic pathways including acting a new master-like regulator of tumor cholesterol biosynthesis program. Importantly, an increasing number of RORγ orthosteric and allosteric ligands are being identified that display potent activities in blocking tumor growth and autoimmune disorders in preclinical models. This review summarizes the recent preclinical and clinical progress on RORγ with emphasis on its role in reprogramming tumor cholesterol metabolism and its regulation. It will also discuss RORγ functional mechanisms, context-specificity and its value as a therapeutic target for effective cancer treatment.

2.2 Abbreviation

ACAT2, acetyl-CoA acetyltransferase 2; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGCS, hydroxymethyl glutaryl-CoA synthase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVK, mevalonate kinase; PMVK, phosphomevalonate kinase; FPP, farnesyl pyrophosphate; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; ABC, ATP-binding cassette; CEs, cholesterol esters; CYP7A1, cholesterol 7α-hydroxylase; SREBP2, sterol regulatory element-binding protein 2; LXRs, liver X receptors; RXR, retinoid X receptor; ER, endoplasmic reticulum; SCAP, SREBP-cleavage activating protein; INSIG, insulin-induced gene; PI3K, phosphatidylinositol 3-kinase; ER+, estrogen receptor-positive; SOAT1, Sterol O-acyltransferase 1; GGPP, geranylgeranyl-diphosphate; OCDO, 6-oxo-cholestan-3β,5α-diol; CRPC, castration-resistant prostate cancer; NR, Nuclear receptor; AR, androgen receptor; RORs, retinoic acid receptor-related orphan receptors; DBD, DNA-binding domain; LBD, ligand-binding domain; Th17, T helper 17 cell; ROREs, ROR response elements; EMT, epithelial-mesenchymal transition; PDAC, pancreatic ductal adenocarcinoma; TNBC, triple-negative breast cancer; PDX, patient-derived xenograft; CBI, cholesterol biosynthesis intermediate; 7-DHC, 7-dehydrocholesterol; 7β, 27-OHC, 7β, 27-dihydroxycholesterol; 7keto, 27-OHC, 7-keto-27-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol; 7α, 27-OHC, 7α, 27-dihydroxycholesterol; 7α, 25-OHC, 7a, 25-dihydroxycholesterol; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 24S-OHC, 24S-hydroxycholesterol; 4ACD8, 4α-carboxy, 4β-methyl-zymosterol; 3-OxoLCA, 3-oxolithocholic acid; 3-oxoCA, 3-oxocholic acid; 3-OxoDCA, 3-oxodeoxycholic acid; VDR, vitamin D receptor; TF, transcriptional factor; PTM, posttranslational modification; IKK, kappa B kinase; AhR, aryl hydrocarbon receptor; PBK, PDZ binding kinase; SAR, structure-activity relationship;

2.3 Introduction

Proper control of cholesterol biosynthesis, metabolism and homeostasis is vital to growth, survival and differentiation of mammalian cells [1]. Cholesterol is a crucial cellular membrane component that controls membrane structural fluidity and forms lipid rafts, which confers important cellular signaling [2]. It is also a precursor to steroid hormones that play important roles in mammalian development and physiological processes such as reproduction and metabolism, and in development and progression of hormone-responsive cancers [3,4]. Intermediate products of mevalonate/cholesterol biosynthesis pathway are also the sources of isoprenoids that are vital to cellular signaling via protein prenylation [5,6].

2.4 Regulation of cholesterol biosynthesis and homeostasis in mammalian cells

In mammalian cells, cholesterol is synthesized through the mevalonate pathway. Initially, two acetyl-CoA molecules condensate by acetyl-CoA acetyltransferase 2 (ACAT2) to form acetoacetyl-CoA; then a third acetyl-CoA join to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by hydroxymethyl glutaryl-CoA synthase (HMGCS). HMG-CoA is then reduced to mevalonate by HMG-CoA reductase (HMGCR), a rate-limiting step of mevalonate/cholesterol biosynthesis pathway. Mevalonate then gets phosphorylated by mevalonate kinase (MVK) and phosphomevalonate kinase (PMVK) to mevalonate pyrophosphate. Through a series of decarboxylation and isomerization reactions, mevalonate pyrophosphate gets converted to an important intermediate farnesyl pyrophosphate (FPP), which is the precursor to isoprenoids. Two FPP molecules can further condensate to squalene, which is committed to sterol formation. Squalene gets converted to epoxysqualene through squalene epoxidase and then lanosterol, which has the four-ring structure. Finally, lanosterol gets converted to cholesterol through additional steps that modifies the four-ring structure [1,7]. To mediate cellular cholesterol homeostasis, mammalian cells can also transport cholesterol in both directions. When cellular cholesterol levels are low, cells can acquire cholesterol by

importing cholesterol-rich low-density lipoprotein (LDL) from the serum via cell surface LDL receptor (LDLR) [8]. On the contrary, when cellular cholesterol levels are higher than demand, the cells express cholesterol transporters belonging to the ATP-binding cassette (ABC) family transporters, including ABC subfamily A member 1 (ABCA1), ABCG1, ABCG5, and ABCG8. Cholesterol can also be esterified to cholesterol esters (CEs) and stored in intracellular lipid droplets, or catalyzed to bile acids by cholesterol 7α-hydroxylase (CYP7A1) and secreted out of the cell [9].

In mammalian cells, cholesterol homeostasis is under tight regulation by transcription factors (TFs), such as sterol regulatory element-binding protein 2 (SREBP2), which plays a predominant role in controlling cholesterol biosynthesis [6] and liver X receptors (LXRs), which function as a master regulator of cholesterol efflux [10]. The wo isoforms of LXRs, LXRα and LXRβ (encoded by NR1H3 and NR1H2, respectively), are members of the nuclear receptor (NR) superfamily of transcription factors. The two LXRs display high sequence homology and respond to the same endogenous ligands, namely sterol metabolites. LXRα is mainly expressed in the liver while LXRβ is universally expressed. When cellular cholesterol and its metabolites levels are high, LXRs are activated by their ligands and form heterodimer complexes with retinoid X receptor (RXR). The LXR/RXR heterodimer binds to LXR-responsive elements (LXREs) in their target genes, including cholesterol efflux transporters ABCA1, ABCG1 and enzyme CYP7A1, and activates their expression [10,11]. When cellular cholesterol falls below a normal level, de novo cholesterol biosynthesis and import are activated by SREBP2. SREBP2 is expressed initially in an inactive form and anchored to the endoplasmic reticulum (ER). It has an N-terminal trans-activation domain and a C-terminal regulatory domain bound by SREBP-cleavage activating protein (SCAP) [12]. SCAP serves as an ER cholesterol level sensor, which can change conformation with cholesterol level fluctuation to modify its binding with COPII-coated vesicles [13]. When ER membrane

cholesterol levels are low, the SCAP-SREBP2 complex is transported to the Golgi via the COPII-coated vesicles, where SREBP2 is subject to enzymatic cleavages by site 1 protease (S1P) and S2P [13]. The resulting N-terminal of SREBP2 then enters the nucleus and activates the transcription of its target genes, including cholesterol biosynthesis gene HMGCR, MVK, and SQLE, and cholesterol import gene LDLR [13,14]. When ER cholesterol levels are high, SCAP associates with insulin-induced gene (INSIG) protein, which blocks its binding with COPII and retains the SCAP-SREBP2 complex in the ER. In addition, cholesterol metabolite oxysterols can directly bind INSIG and promote its association with SCAP [15]. Increased levels of certain oxysterols can also activate LXRs to enhance cholesterol efflux. The concerted actions of decreased biosynthesis and increased efflux will maintain cellular cholesterol homeostasis.

The question whether retinoic acid receptor-related orphan receptor gamma (RORγ), another member of the NR family, directly regulates cholesterol biosynthesis and/or metabolism in normal mammalian tissue or cells is currently unclear. In this regard, findings from two lines of studies strongly implicate such a role for RORγ. The first is mouse RORγ gene knockout studies which showed that the expression of INSIG2 was reduced in the liver and that cholesterol levels in liver and serum were decreased in the knockout mice [11,12]. The other is studies with different approaches demonstrating that cholesterol biosynthesis intermediates (CBIs) and oxysterols can act as endogenous ligands of RORγ, which will be described in more details in section 5.

2.5 Deregulation of cholesterol biosynthesis and homeostasis in cancer cells

Cancers cells are generally fast proliferating and thus require a relatively high level of cholesterol not only for membrane formation but also for cellular signaling. Membrane lipid rafts are hubs for signaling by different families of proteins such as receptor tyrosine kinases (e.g. EGFR), phosphatidylinositol 3-kinase-protein kinase Akt (PI3K-AKT), CD44, cell death

receptors and mucin 1, which play critical roles in cancer cell growth, survival, invasion and metastasis [2,16]. Cholesterol is one of the major components of lipid rafts. It promotes lipid raft formation by facilitating packing of phospholipids and phase separation. Prostate cancer and breast cancer cells are found to contain elevated levels of lipid rafts. Disruption of these membrane rafts would result in inhibition of cell growth [17-19]. Moreover, in steroid-responsive tumors such as those of prostate cancer and estrogen receptor-positive (ER+) breast cancer, cholesterol is the precursor of different steroid hormones such as androgens and estrogens which are de novo synthesized by prostate cancer and breast cancer cells respectively and are major promoters of cancer progression [20,21]. As a result, aberrant cholesterol metabolism is a major contributor to various types of cancer progression, making it a potential therapeutic target [22].

Increasing evidence from clinical and experimental studies clearly indicates an important role of aberrant cholesterol metabolism in cancer development and progression. In prostate cancer, high levels of circulating cholesterol are positively associated with the development of high-grade prostate cancer [23]. Use of statins, drugs that are prescribed for patients with hypercholesterolemia to lower circulating levels of cholesterol, was found to associate significantly with a decreased risk in prostate cancer death or with longer time to progression [24,25]. Analysis of patient tumors also revealed that de novo cholesterol biosynthesis is a key characteristic of lethal prostate cancers. The mRNA of SQLE, a major enzyme in the pathway, is highly elevated in the tumors and is positively correlated with high Gleason grade and the lethal form of prostate cancer [26,27]. On the other hand, lower mRNA levels of cholesterol uptake gene LDLR and cholesterol esterification enzyme SOAT1 appear to associate with the disease lethality [27]. Besides prostate cancer, genes of de novo cholesterol biosynthesis pathway is also found to be upregulated in breast cancer [28], glioblastoma multiforme [29], colon carcinoma [30], and pancreatic adenocarcinoma [31].

With the upregulated cholesterol biosynthesis pathway, production of CBIs in the pathway and cholesterol metabolites downstream may also be deregulated in the tumors. Their abnormal levels can also promote cancer progression. For example, FPP and geranylgeranyl-diphosphate (GGPP) are involved in protein prenylation, which attaches proteins to the cell membrane to facilitate their localization and cellular function [31,32]. Protein prenylations are involved in cellular signaling by the G-protein coupled receptor (GPCR) and small GTPases (e.g. RAS and RHO) [33], which are important players in cancer development and progression [34,35]. Moreover, accumulation of cholesterol metabolites such as CEs and oxysterols is another common characteristic in cancer. CE is found to promote pancreatic cancer and lymphocytic leukemia progression [36,37]. Inhibition of CE catalyzing enzyme sterol O-acyltransferase 1 (SOAT1, as known as ACAT1) can significantly inhibit cancer progression, including prostate cancer [38], breast cancer [39], and glioma models [40]. Mechanistically, loss of tumor suppressor PTEN in high grade prostate cancer results in activation of PI3K/AKT, which further leads to accumulation of CE. CE accumulation can promote prostate cancer tumor growth and invasion [38] and may also stimulate secretion of Wnt3a, an important signaling factor of Wnt/β-catenin pathway [41]. In addition to CE, cholesterol metabolites oxysterols may also be potential cancer promoters. For example, 27-hydroxycholesterol (27-OHC) is elevated in ER+ breast cancer, which promotes tumor growth and metastasis to the lung [42,43]. It is also found to suppress tumor suppressor p53 activation [44] and promote angiogenesis by activating VEGF signaling [45]. Interestingly, in prostate cancer, CYP27A1 was identified as one of the most down-regulated genes involved in cholesterol metabolism [46]. CYP27A1 encodes sterol 27-hydrolase that converts cholesterol to 27-OHC. Treatment of prostate cancer cells in vitro with 27-OHC inhibited the activation of SREBP2 and decreased cellular cholesterol content. These results together suggest that tumors can adopt a mechanism to silence the expression of certain cholesterol metabolism and efflux genes such as CYP27A1

and ABCA1 to raise the tumor cholesterol content [46,47]. Interestingly, certain cholesterol metabolites were demonstrated to possess either pro-tumorigenic or anti-tumorigenic activities [48,49]. Cholesterol can be epoxidated to 6-oxo-cholestan-3β,5α-diol (OCDO) by cholesterol epoxide hydrolase (ChEH) which has 3β-hydroxysteroid δ8-δ7-isomerase (D8D7I, also known as EBP) as its catalytic subunit and the 3β-hydroxysteroid δ7 reductase (DHCR7) as its regulatory subunit. Interestingly, OCDO level was found to be significantly elevated in breast cancer tumors and OCDO stimulates ER+ breast cancer cell growth via acting through glucocorticoid receptor (GR) [50].

In addition to its biosynthesis and metabolism, efflux of cholesterol is also dysregulated in several types of cancer that may contribute to tumor progression. For example, tumor suppressor TERE1 has been shown to regulate cholesterol efflux in castration-resistant prostate cancer (CRPC). Loss of TERE1 resulted in decreased levels of vitamin K-2, which is a potent LXR agonist, thus downregulating LXR transcription activation and its target genes. As a result, cholesterol efflux rate decreased in CRPC cells and cholesterol levels built up to allow more endogenous androgen synthesis [51]. Also, in prostate cancer cells, cholesterol efflux was downregulated by loss of ABCA1, the major cholesterol transporter and target gene of LXR. ABCA1 gene was found to be hypermethylated, leading to decrease in its expression and increase in intracellular cholesterol levels [47]. Also, loss-of-function mutations in ABCA1 promoted colon cancer cell survival [52]. ABCA1 expression has also been found to be inversely related to aggressive phenotype in breast cancer [53]. Dysregulation of another important cholesterol transporter ABCG1 was also found to associate with progression of lung and prostate cancer [54,55]. Pharmacological agonism of LXR induced apoptosis in breast cancer cells via increased expression of ABCG1 transporter [56]. Activation of LXR by its agonist T0901317 decreased intratumoral cholesterol levels by upregulating ABCG1 in prostate cancer cells, resulting in disrupted lipid raft dependent AKT survival signaling

pathway, thus induced apoptosis [57]. In summary, different cancer types display various dysregulation of cholesterol efflux, pointing to therapeutics targeting of targets such as LXRs as potentially effective strategy in treating tumors with cholesterol pathway aberration.

In tumor cells, in addition to LXRs, several other TFs and signaling pathways play major roles in the deregulation of cholesterol metabolism [6,7]. The expression and function of SREBP2 as well as SREBP1 are often hyper-activated by oncogenic PI3K-AKT and mTORC1 signaling. Oncoprotein MYC functionally interacts with SREBPs to promote their transcriptional activities in cholesterol biosynthesis. Interestingly, p53 protein with gain-of-function mutations can also interact with SREBP2 to stimulate the mevalonate pathway. As described in more details below, recent studies revealed that RORγ can play an important role in cholesterol biosynthesis in tumor cells of specific cancer types.

2.6 RORγ, RORγt and their functions in metabolism, immunity and cancer

RORγ and RORγt

NRs comprise a superfamily of structurally conserved, ligand-regulated transcription factors which include steroid hormone receptors such as androgen receptor (AR) and estrogen receptor α and β, and non-steroid receptors such as aforementioned LXRs and RORγ [58]. Aberrant function of NR signaling leads to proliferative, reproductive and metabolic diseases as well as specific types of cancer such as prostate cancer and ER+ breast cancer [59,60]. Recent studies have uncovered important roles of RORs in control of metabolism, circadian rhythm and in cancer [61-65]. The RORs subfamily has three members—RORα, β and γ (Figure. 2.1) which are encoded respectively by RORA, RORB and RORC genes and display distinct expression patterns. RORα and RORγ are widely expressed in a variety of tissues, including kidney, lung, liver, skeletal muscle, thymus, prostate and adipose tissue. RORβ has a restricted pattern of expression in certain regions of the central nervous system (CNS), retina and pineal gland [61,66]. Like other NRs, the three RORs share high structural

similarities in DNA-binding domain (DBD) and the carboxy-terminal ligand-binding domain (LBD) [66,67]. Potential natural ligands of RORs identified thus far include steroids, terpenoids, polyketides and cardiac glycosides [68]. Ligand binding to ROR LBD leads to a conformational change in the receptor and recruitment of other co-regulatory proteins, affecting the transcriptional activity of RORs. However, except for certain cholesterol intermediates and oxysterols as described below, the precise physiological role and action mechanism of the other potential natural ligands in control of RORs are yet to be established.

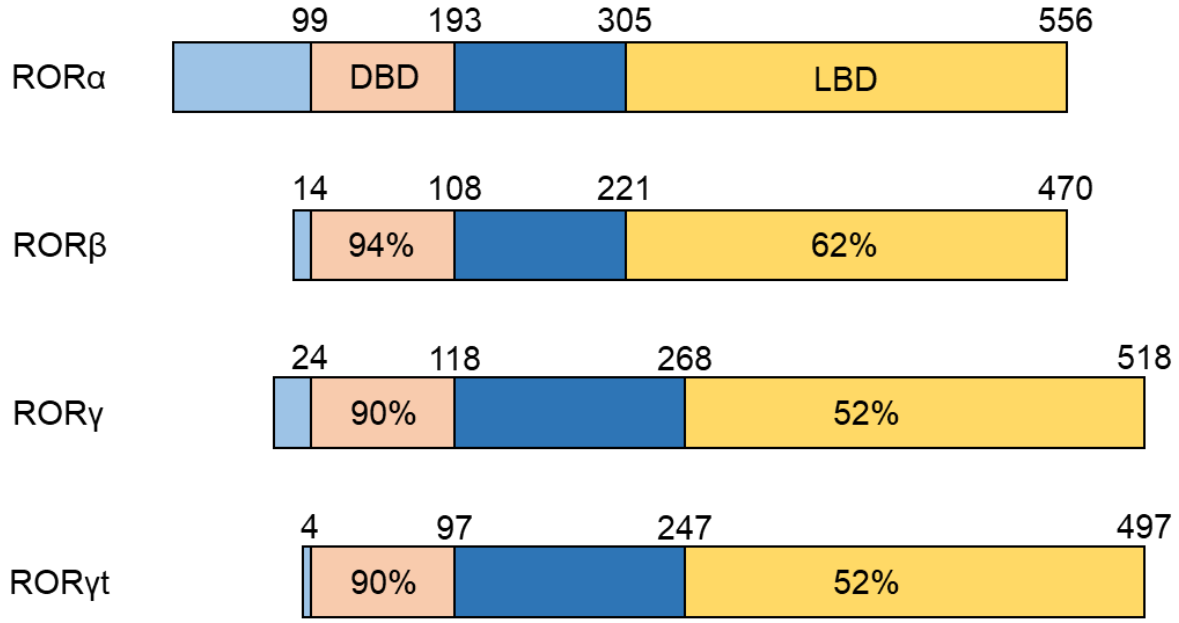


Figure 2.1 Diagram of domains of the retinoic acid receptor-related orphan receptors (RORs). Numbers above each RORs indicate the amino acid positions at the junction of different domains. Protein sequences displayed here are from the following NCBI reference sequences: RORα, NP_599022.1; RORβ, NP_001351952.1; RORγ, NP_005051.2; RORγt, NP_001001523.1. Percentages indicate amino acid identity within a particular domain relative to RORα. DBD, DNA-binding domain; LBD, ligand-binding domain.

Various biological processes have been reported to be regulated by RORs, including circadian rhythm, metabolism and immune functions [61,62,64]. Due to its critical connection to many auto-immune and inflammatory diseases, including multiple sclerosis and rheumatoid arthritis [69,70], RORγ is one of the most studied members in RORs. RORγ has two isoforms,

RORγ1/RORγ and RORγ2/RORγt (Figure. 2.1), which are translated from mRNAs that are transcribed from alternative promoters. Therefore, they display distinct expression patterns in a tissue-specific manner. RORγ1/RORγ is widely expressed in many tissues, while RORγt is exclusively highly expressed in thymus and sub-populations of immune cells [61,62]. RORγ displays an oscillatory expression in liver and pancreatic β cells [64,71,72] and regulates circadian rhythm gene expression [64,73,74]. RORγ also plays a key role in gluconeogenesis, insulin sensitivity as well as lipid metabolism [75,76], whereas RORγt is critical in T helper 17 cells (Th17) differentiation, innate lymphoid cell (such as ILC3) development and functions of γδ T cells [77-79].

Functions of RORγ and RORγt in metabolism and immune system

RORγ has been found to be involved in the control of several metabolic pathways, including gluconeogenesis, lipid metabolism and sterol metabolism [75,76,80,81]. RORγ gene RORC knockout mice showed decreased hepatic gluconeogenesis, leading to improved insulin sensitivity and glucose tolerance. ChIP-seq analysis of mouse liver tissue demonstrated that RORγ directly binds to the regulatory regions of glucose metabolism genes, including G6PC, SLC2A2, GCK, GCKR, KLF15, PPARD, PKLR and GYS2. Loss of RORγ reduced the expression of these genes in a ZT-dependent manner [75]. Exogenous over-expression of RORγ in skeletal muscle cells increased expressions of several genes involved in lipid metabolism, such as SLC2A5, ADIPOR2, IL-15 and MSTN [76]. In addition, RORγ directly controls lipid metabolic gene INSIG2A and ELOVL3. RORγ knockout mice exhibited a reduced expression level of INSIG2A, ELOVL3 and CYP8B1, resulting in decreased cholesterol and bile acids levels in both liver and serum [80,81]. Decreased INSIG2 expression can then further activate lipogenesis through activation of SREBP1 [81].

The well-studied role of RORγt is its regulation of Th17 cell differentiation [77]. RORγt expression can be upregulated by IL-6 and TGF-β [63,77,82]. Although not necessary for Th17

differentiation, IL-23 maintains the differentiated state by stimulating RORγt expression via inducing expression of Runx1 [83]. Induced RORγt can then bind to ROR response elements (ROREs) on various Th17 differentiation-associated gene loci, including IL-17A, IL-17F and IL-23R, activating their transcription [63]. The differentiation of regulatory T cell (Treg) or Th17 lineages also relies on the relative abundance of FOXP3 and RORγt [84-86]. Other than Th17 cells, RORγt also plays an important role in innate lymphoid cells. It is selectively expressed in ILC3 cells. RORγt is regulated by Runx3 and in turn directly regulate aryl hydrocarbon receptor (AHR) gene, which is required for the development and function of ILC3 cells [87]. Moreover, RORγt-deficient mice fail to develop secondary lymphoid organs [62]. In addition, γδ T cells also express IL-17 in a RORγt-dependent manner and is involved in several autoimmune diseases [88,89].

Functions of RORγ in cancer

Research on the role of RORγ in cancer did not begin until recent years. Through a meta-analysis of NR gene expression profile in prostate cancer data sets followed by IHC analysis, a study in 2016 found that the expression level of RORγ is higher in metastatic tumors when compared with benign prostate tissue or primary prostate tumors, while RORα and RORβ appeared to have lower expression in the metastatic tumors [90]. RORγ gene knockdown and treatment with its antagonists caused CRPC cell apoptosis and inhibited the cell proliferation and survival. RNA-seq analysis showed that AR signaling pathway is controlled by RORγ, and that AR-signature gene expression was downregulated by RORγ antagonists. Further investigation using ChIP-seq revealed that RORγ inhibition diminished AR chromatin binding and altered genome-wide H3K27ac histone modification. Additional ChIP assays confirmed that RORγ directly binds to an RORE in the first exon region of AR gene and regulates the expression of full-length AR as well as its variant forms including AR-V7. Together these results demonstrated that RORγ plays a crucial role in control of bother AR gene

overexpression and AR tumorigenic signaling in prostate cancer (Figure 2.2). Interestingly, the role of RORγ in AR expression was not observed in non-malignant, human prostate epithelial cells. Moreover, RORγ antagonists alone or combined with AR inhibitor enzalutamide significantly inhibited CRPC tumor growth and metastasis in the xenograft tumor models [90].

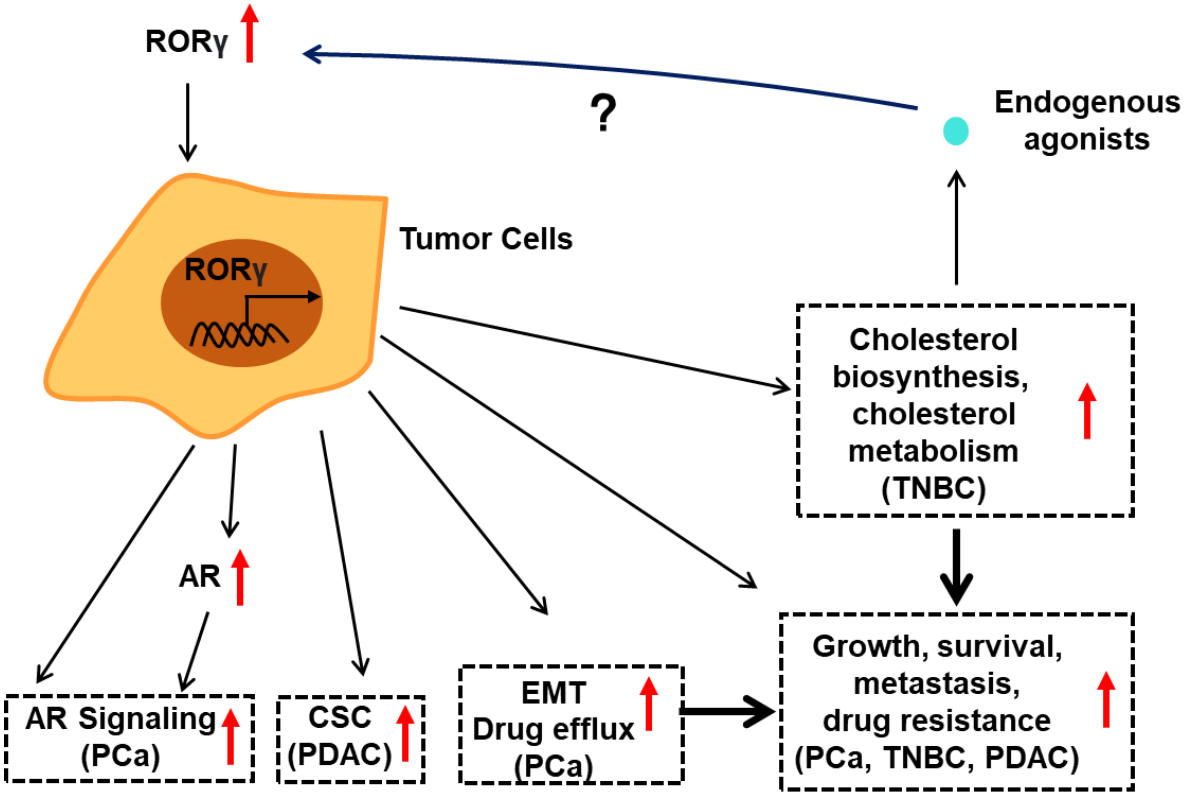


Figure 2.2 A simplified illustration of RORγ functions in the different types of cancer. Note: "?" denotes a postulated feedforward loop in tumor cells where RORγ increases the level of specific cholesterol intermediates and/or metabolites, which in turn bind to RORγ and further enhance its function in stimulating the metabolic pathways and activating the pro-tumorigenic programs. PCa, prostate cancer; TNBC, triple-negative breast cancer; PDAC, pancreatic adenocarcinoma; AR, androgen receptor; CSC, cancer stem-like cells; EMT, epithelial mesenchymal transition.

In addition to control of AR signaling in prostate cancer, a recent study [91] demonstrated that RORγ controls the expression of MDR1/ABCB1, which is one of the major mediators of chemotherapy drug taxane resistance in CRPC cells. It was also shown that RORγ antagonists re-sensitized taxane-resistant CRPC cells and tumors to taxanes in an AR-independent manner.

Interestingly, RORγ was found to be highly elevated in doxorubicin-resistant CRPC cells to promote resistance to the chemotherapy drug [92]. Another recent study showed that RORγ can regulate genes of epithelial-mesenchymal transition (EMT) in CRPC cells [93]. PBK, one of the EMT-related signature genes was identified as a downstream target of RORγ. RORγ positively regulates the expression of PBK which in turn stabilizes RORγ and AR proteins. Together with previous findings that RORγ activates AR gene, thes recent results support a model where RORγ, PBK and AR form interlocked feedforward loops in enforcing the hyperactive signaling by AR and RORγ in CRPC [93]. Moreover, pharmacological inhibition of both RORγ and PBK synergistically inhibited the expression and function of AR and AR-V7, leading to a strong inhibition of the growth of CRPC cells and tumors [93].

Pancreatic cancer is one of the deadly cancer types due to a lack of effective therapies. RORγ was recently identified as an important player in pancreatic cancer [94]. Based on a genome-wide CRISPR screening, RORγ was reported as necessary for pancreatic cancer stem cell growth and survival. RNA-seq also showed it has a higher expression in cancer stem cells than non-stem cells. Its expression level increased dramatically in mouse tumor epithelial cells relative to normal pancreatic cells. Knockdown of RORγ led to increase in cell death, decrease in cell proliferation and depletion of stem cells. RNA-seq analysis suggested that RORγ may control gene programs of stem cell and tumorigenesis. Pharmacological inhibition of RORγ alone or combined with chemo-drug in tumor-bearing, immunocompetent mice significantly reduced tumor growth, depleted stem cell burden and improved survival. Similar effect was observed in immuno-compromised mice, indicating the anti-tumor activity of RORγ inhibitor is tumor cell-specific and Th17- and other immune cells-independent. Moreover, RORγ mRNA expression was found to positively correlate with aggressiveness of pancreatic ductal adenocarcinoma (PDAC) [94].

TNBC is an aggressive cancer subtype, largely due to its lack of effective therapeutic targets such as estrogen receptor α (ERα), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2). In a recent study, RORγ was demonstrated as an attractive therapeutic target and a master regulator of tumor cholesterol biosynthesis in TNBC [95]. First, meta-analysis of breast cancer tumor datasets revealed that the expression of RORγ gene is significantly associated with the poor survival of TNBC patients but not with ER+ breast cancer patients. Further analysis indicated that high levels of RORγ expression correlates with the expression of cholesterol biosynthesis genes in TNBC tumors but not in ER+ tumors. CRISPR knockout, siRNA and pharmacological inhibition of RORγ caused strong inhibition of TNBC cell growth and survival, whereas its knockout or antagonists had marginal effects on ER+ breast cancer cells and non-tumorigenic epithelial cells, suggesting that RORγ functions selectively in a subtype of breast cancer. Treatment of tumors with RORγ inhibitors alone or in combination with cholesterol lowering drug statin led to strong tumor regression and blocking of metastasis in multiple models including patient-derived xenograft (PDX) models and an immunocompetent mouse model. Further investigations revealed that one major mechanism of RORγ function in TNBC is its direct control of cholesterol biosynthesis program [95], as described below.

Function of RORγ in control of tumor cholesterol metabolism

As described in more details in the section below, certain intermediates of cholesterol biosynthesis pathway can act as potent, agonistic or antagonistic ligands of RORγ, therefore implicating a potential role of RORγ in control of cholesterol metabolism. However, direct evidence was lacking for such a role until a recent study [95]. In its initial pharmacological interrogation of different nuclear receptors in control of the expression of key cholesterol biosynthesis gene program in breast cancer cells, the study found that only two antagonists (XY018 and GSK805) of RORγ consistently suppressed the expression of key cholesterol

biosynthesis genes such as HMGCS1, HMGCR, SQLE, MVK and MVD in a TNBC cell line. Interestingly, the antagonists did not display any strong effect on the genes in the ER+ breast cancer cells. In agreement with the established function of LXRs in control of cholesterol intake, agonists of LXRs such as GW3965 and LXR623 strongly induced the expression of cholesterol efflux gene ABCA1 in both the TNBC and ER+ cells. Further analyses revealed that knockdown of RORγ or treatment with the antagonists at low μM suppressed the mRNA and protein expression of the majority of the 21 cholesterol biosynthesis genes in the TNBC cells and a PDX tumor, with HMGCS1, HMGCR, MVK and SQLE being inhibited more pronouncedly than the other genes. Such a prominent function of RORγ appeared to echo the role of SREBP2. Indeed, RORγ knockdown and its antagonists significantly reduced the cellular cholesterol content in the TNBC cell culture and in the xenograft tumors. Measurement of cholesterol biosynthesis rate by GC-MS revealed that inhibition of RORγ by antagonist XY018 significantly decreased the rate of cholesterol biosynthesis in the tumors [95].

The cholesterol master regulator SREBP2 directly controls the majority of cholesterol biosynthesis genes as revealed by ChIP-seq with mouse liver tissues [96,97] and reporter gene assays [98,99]. In the TNBC cells, ChIP-seq analysis demonstrated SREBP2 binding to promoter region of most of the 21 genes except HSD17B7 [95], suggesting that the master function of SREBP2 is largely conserved in cancers such as TNBC. Remarkably, in the same study, RORγ ChIP-seq analysis revealed that RORγ binds to sites adjacent to SREBP2 binding sites in the promoter region of most of the cholesterol biosynthesis genes. Further studies demonstrated that RORγ interacts with SREBP2 and that the interaction between the two proteins can be diminished by the RORγ antagonist. Histone acetylase p300 is a coactivator of both RORγ and SREBP2 and a major epigenetic writer of H3K27ac mark. Interestingly, the occupancy of p300 and the H3K27ac mark at the promoter region of cholesterol biosynthesis genes were strongly inhibited by the RORγ antagonist [95], suggesting a critical role of RORγ

in mediating transcriptional activation of the gene program through recruitment of histone acetylase p300 (Figure 2.3).

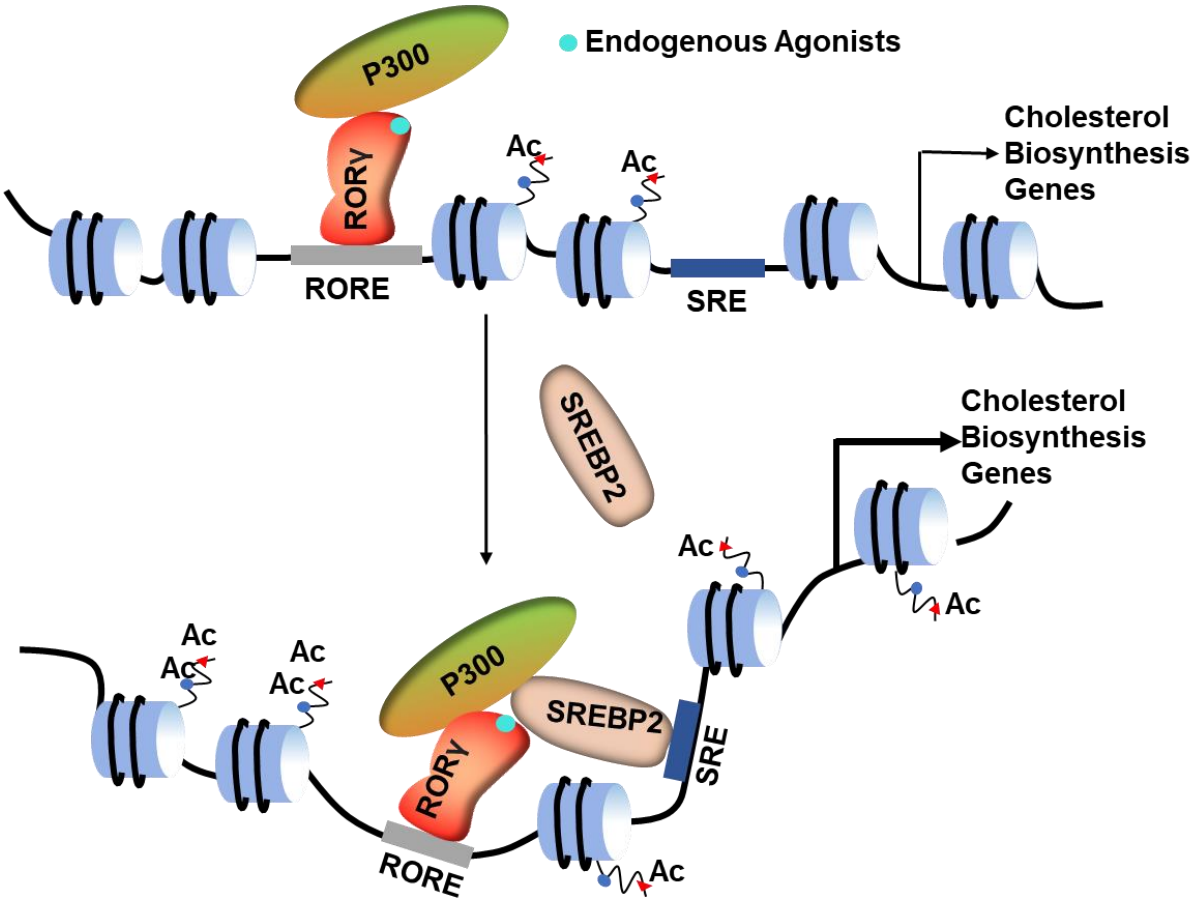


Figure 2.3 A hypothetic model depicting the functional mechanisms of RORγ in control of tumor cholesterol biosynthesis program. Ac, H3K27ac mark; RORE, ROR response element; SRE, sterol regulatory element.

One striking observation made in the recent study [95,100] is that in the TNBC cells RORγ antagonist XY018 markedly reduced genome-wide SREBP2 binding without altering its protein expression. The impact of XY018 was particularly pronounced at the cholesterol biosynthesis genes. On the other hand, the inhibitor did not significantly alter the binding of RORγ either at genome-wide or at the cholesterol biosynthesis genes, which is in line with other studies that RORγ antagonists can inhibit the receptor function without significantly perturbing its binding to the targets [101]. Intriguingly, activation of cholesterol biosynthesis genes by the constitutively active/nuclear form of SREBP2 was strongly inhibited by the RORγ

antagonist whereas stimulation of those genes by ectopic RORγ was not affected by SREBP2 knockdown, suggesting that RORγ acts as a key determinant in the SREBP2 function. Together, these findings support a model that in TNBC cells RORγ interacts with SREBP2 and facilitates the recruitment of SREBP2 to its chromatin targets, and that under certain conditions RORγ acts dominantly over that of SREBP2 (Figure 2.3).

Currently, it is unclear to what extent the physical and functional interplays between RORγ and SREBP2 operate in other cancer types or normal tissues. Recent studies in this laboratory confirmed a crucial role of RORγ in control of several genes in cholesterol biosynthesis program in prostate cancer cells. However, studies of RORγ in pancreatic cancer did not show any significant role of RORγ in control of cholesterol gene programs [94]. As to the role of RORγ in normal tissues, an earlier study with RORC KO mice revealed that liver-specific deletion of RORC significantly reduced liver and serum levels of bile acids, triglycerides and cholesterol in mice fed with high fat diet (HFD). Its ChIP-seq and qRT-PCR analyses showed that RORγ plays an important role in direct activation of lipid metabolic genes such as INSIG2, ELOVL3 and CYP27A1. However, any potential role of RORγ in direct control of liver cholesterol biosynthesis and metabolism was not reported [80]. INSIG2, like INSIG1, is an endoplasmic reticulum (ER) protein. Their up-regulation attenuates the activation of SREBPs via retaining SCAP in the ER and thus preventing SREBP translocation to the Golgi for processing to the active form. Thus, in the liver, RORγ may be involved in maintaining the homeostasis of cholesterol by fine-tuning of cholesterol biosynthesis, unlike the predominant function in TNBC where it activates almost the entire program of cholesterol biosynthesis. Studies of RORγt in immune cells led to the elucidation of a critical role of RORγt in differentiation and function of T cell subtypes including Th17, ILC3 and a subset of γδ T cells [63]. Recent studies uncovered that certain intermediates and metabolites of cholesterol metabolisms (details are described below) can serve as RORγt modulators. However, the

question whether RORγt directly controls cholesterol metabolism programs is yet to be addressed. Overall, it is conceivable that the function of RORγ in control of cholesterol biosynthesis and metabolism may be tissue-specific in that in some tissues such as TNBC RORγ can play a master-like role in cholesterol biosynthesis whereas in other tissues it may act as an important modulator of cholesterol homeostasis.

In summary, recent research has revealed a multi-facet role of RORγ in cancer, which include a SREBP2 master-like function in control of tumor cholesterol biosynthesis, its regulation of key cancer drivers such as AR, major tumorigenic pathways such as the AR signaling pathway, cancer stem cell program, EMT, and therapeutic resistance (Figure 2.2). Interestingly, its function in activation of cholesterol biosynthesis appears to be most prominent in specific subtypes of cancer such as TNBC.

2.7 Regulation of RORγ function

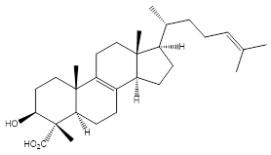
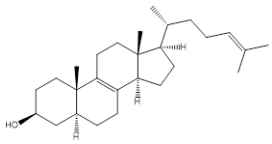
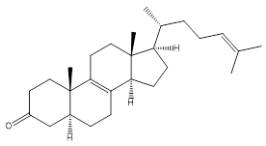
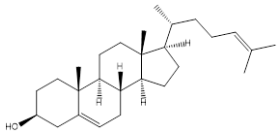
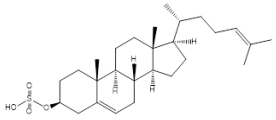
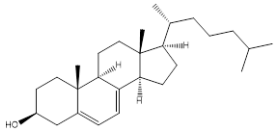
Cholesterol biosynthesis intermediates (CBIs), oxysterols and bile acids as endogenous ligands of RORγ

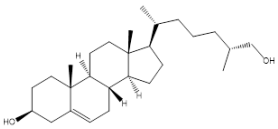
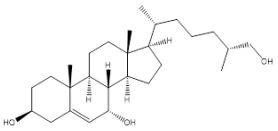
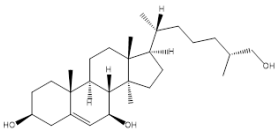
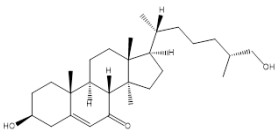
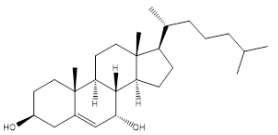
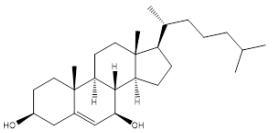
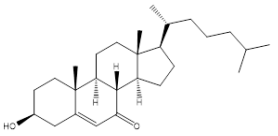
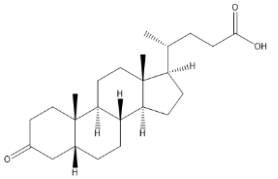
RORγ and its related subfamily members, RORα and RORβ, were identified initially as orphan nuclear receptors without any known physiologically relevant ligands [61]. The strong transcriptional activation activity displayed by RORγ in reporter gene assays with mammalian cells in the absence of an exogenous ligand supported the notion that RORγ may act as a constitutive activator independent of any ligand binding-invoked regulation. In line with this notion, X ray crystallography studies showed that, without ligand binding, the LBD of RORγ adopts a transcriptionally active conformation with its C-terminal helix 12 positioned for recruiting coactivator peptides [102]. One key structural element appears to be a hydrogen bond formed between His479 in helix 11 (H11) and Tyr502 in helix 12 (H12) which may keep the LBD in the active conformation and is often referred to as His-Tyr lock. However, further structural studies revealed that the major helices of LBDs (e.g. H3, H5, H6, H7, H11 and H12)

of RORs form specific pocket structures with a relatively large size that would allow high affinity interactions with small molecules with different structures. Indeed, RORγ as well as RORα was found to bind to specific cholesterol metabolites such as cholesterol sulfate and hydroxysterols [103]. The bindings in RORγ involve amino acid residues Q286 and L287 in interactions with the 3β-hydroxy group of oxysterols or the carboxy group of CBI 4α-carboxy, 4β-methyl-zymosterol or 4ACD8 [103]. Using in vitro binding assay and reporter gene assays performed in mammalian and insect cells, in combination with knockdown of specific cholesterol biosynthesis genes, several studies identified additional CBIs as likely endogenous ligands of RORγ, which include zymosterol, zymosterone, 7-DHC and desmosterol whereas cholesterol, lanosterol and T-MAS displayed only weak agonist activity [103,104] (Table 2.1). Among the oxysterols, 7β, 27-dihydroxycholesterol or 7β, 27-OHC, 7keto, 27-OHC, 27-OHC and 7α, 27-OHC (listed in the order of efficacy) appeared to possess strong activity in further activating RORγ [105]. Other oxysterols such as 7α, 25-OHC, 7α-OHC, 7β-OHC, 25-OHC, and 24S-OHC were either inactive or weakly active, as demonstrated in the above studies. Although several sulfated sterols such as 25-OHC sulfate, desmosterol sulfate and 5α, 6α-epoxycholestanol sulfate showed high affinity binding to RORγ LBD, only desmosterol sulfate appeared to show strong biological activities in stimulation of RORγ functions [104]. For the biological significance of the endogenous ligands identified, the studies have all focused on their role in modulation of the RORγt function in Th17 cell differentiation and/or the production of IL-17A. In each case, the agonistic ligands such as 4ACD8, desmosterol, zymosterol, 7β, 27OHC and 7α, 27-OHC significantly enhanced the differentiation of Th17 cells. In agreement, genetic depletion of genes (e.g. MSMO1, TM7SF2, SC5D) encoding enzymes responsible for producing the different ligands strongly impaired Th17 cell development [103-105]. On the oxysterols, in addition to CYP27A1, a key enzyme that

produces 27-OHCs, 11β-hydroxysteroid dehydrogenase (11β-HSD) has also been shown to regulate RORγ by controlling its access to 7β, 27-OHC [105,106].

Table 2.1 Natural ligands of RORγ and its related receptors

Name	Structure	Precursor	Catalytic enzyme	Receptor bound	Ligand type	Ref.
4ACD8		T-MAS	SC4MOL	RORα RORγ	RORα: unknown RORγ: agonist	[103]
Zymosterol		zymosterone	HSD17B7	RORγ	agonist	[103]
Zymosterone		4ACD8	NSDHL	RORγ	agonist	[103]
Desmosterol		7-dehydrodesmosterol	DHCR7	LXR RORγ	agonist	[104,166]
Desmosterol sulfate		desmosterol	SULT2B1	RORα RORγ	agonist	[104]
7DHC		lathosterol	SC5D	RORα RORγ	agonist	[103,167]

27-OHC		cholesterol	CYP27A1	GPR183 ER LXR RORγ	ER: selective modulator GPR183, LXR, RORγ: agonist	[105,168-170]
7α, 27-OHC		27-OHC 7α-OHC	CYP7B1 CYP27A1	GPR183 RORγ	agonist	[105,168]
7β, 27-OHC		7β-OHC	CYP27A1	GPR183 RORγ	agonist	[105,168]
7keto, 27-OHC		7-ketocholesterol	CYP27A1	RORγ	agonist	[105]
7α-OHC		cholesterol	CYP7A1	GPR183 RORα RORγ	GPR183: agonist RORs: Inverse agonist	
7β-OHC		cholesterol	autoxidation	GPR183 RORα RORγ	GPR183: agonist RORs: Inverse agonist	
7-ketocholesterol		cholesterol	autoxidation	RORα RORγ	Inverse agonist	
3-OxoLCA		LCA	CYP3A4	GP-BAR1 VDR FXR PXR RORγ	GP-BAR1, VDR FXR, PXR: agonist RORγ: Inverse agonist	

In addition to the oxysterols mentioned above, certain oxysterols in the bile acid biosynthesis pathways have also been strongly suggested as endogenous ligands of RORγ (Table 2.1). 7-OHCs such as 7α, 7β, or 7keto-OHC can bind to RORα and RORγ with high affinities and interestingly suppress the transactivation by RORγ [107]. 7α-OHC is the product of CYP7A1, the first and rate-limiting enzyme of bile acid biosynthesis in cholesterol metabolism. Moreover, in a screen of primary and secondary bile acids that can modulate Th17 cell differentiation, a recent study showed that 3-OxoLCA can directly bind RORγ and inhibit its transcriptional activity whereas other bile acids such as 3-OxoCA and 3-OxoDCA possess much weaker activities than 3-OxoLCA [108]. Feeding a strain of mice that contain a relatively high population of Th17 cells in their small intestine with chow containing 0.3% 3-OxoLCA for a week was sufficient to decrease the Th17 cell population. Of note, 3-OxoLCA was previously identified as a potent activator of vitamin D receptor (VDR) [109]. Bile acids-activated VDR can also play an important role in the induction of a subtype of Treg immune cells that express both FoxP3 and RORγt in the intestine [110]. Although it is currently unclear how 3-OxoLCD is produced and whether 3-OxoLCA and RORγt is involved in the subtype of Treg cell development, these recent findings highlight the importance of bile acid-derived oxysterols in regulation of RORγt and other NR's function in mammalian immune system.

Currently, the role of CBIs, oxysterols or bile acids in control of RORγ function in other normal tissue or in cancer is largely unknown. CBIs such as desmosterol and oxysterols such as 27-OHC are endogenous activating ligands of LXRs [11]. Interestingly, 27-OHC which is relatively abundant in circulation can also act as an agonist of ERα to stimulate tumor growth of ER+ breast cancer [42,111,112]. Recent studies show that plasma levels of certain oxysterols such as 7-OHC and 27-OHC are associated with tumor clinicopathology such as tumor size and the survival of breast cancer patients [113]. Given tumor heterogeneity and complex tumor microenvironment, an accurate measurement of CBIs and oxysterols in tumor tissues is

technically challenging. On the other hand, studies have shown that specific enzymes and genes responsible for their production can be particularly important in growth and metastasis of certain subtypes of cancer. MSMO1/SC4MOL that produces 4ACD8 and HSDHL for zymosterol were found to be critical for EGFR signaling in skin and lung cancer cells [114]. NSDHL is also shown to be a potential driver of TNBC metastasis [115]. Genes for CBIs such as MSMO1, TM7SF2, SC5D and DHCR7 were elevated in AR-driven CRPC bone metastasis [116]. Given that the expression and function of RORγ is likely elevated in metastatic tumors [90,95], it is conceivable that certain CBIs and oxysterols might play an important role in regulation of RORγ in tumor growth and metastasis in specific subtypes of cancer, in addition to their activities in modulation of the other NRs (e.g. LXRs and ERα). Future investigations to define the role of tissue-endogenous ligands in control of RORγ in non-immune cells including cancer cells will provide insights to better understanding of RORγ functions in circadian rhythm, metabolism and cancer.

Other major mechanisms of regulation of RORγ function

One well established mode of NR function in gene activation is through association with the three NCOA co-activators (also known as SRC1-3) and/or the lysine acetyltransferases such as p300 and CBP [65,117]. In prostate cancer cells, RORγ recruits NCOA1 and NCOA3 to the RORE site at the AR gene. Knockdown experiments demonstrated that NCOA1 and NCOA3, but not NCOA2, play a crucial role in mediating the AR gene expression [90]. In TNBC cells, ChIP assay showed that p300 is recruited to the promoter regions of cholesterol biosynthesis genes such as HMGCS1, MVK, and SQLE although it is unclear whether the NCOAs are involved in the RORγ function [95].

Like other TFs, RORγ or RORγt can be subject to posttranslational modifications (PTMs) such as phosphorylated, ubiquitinated, acetylated and sumoylated [63,118]. However, most of the studies on the biological role of RORγt PTMs were performed in the context of mouse immune

cells [118-123]. The inhibitor of kappa B kinase (IKK) family members such as IKKα and IKKβ phosphorylates a number of transcription factors such as NFκ-B and IRF3/4 to regulate inflammation and innate immunity [124]. One study demonstrated by mass spectrometry that RORγt in Th17 cells are phosphorylated at multiple sites. It also showed that IKKα phosphorylates S376 of mouse RORγt, which enhances its function in Th17 differentiation whereas phosphorylation of RORγt at S484 by an un-identified kinase reduces the RORγt function [125]. Interestingly, another study found that IKKβ phosphorylates S489 of mouse RORγt and that the phosphorylation promotes RORγt interaction with and transportation by aryl hydrocarbon receptor (AhR) [126], another major TF that is involved in IL-17A induction [127,128].

Several studies revealed the role of RORγt ubiquitination in regulation of its function in Th17 cells. RORγt can be K48-ubiquitinated by ITCH, a HECT E3 ubiquitin ligase, which promotes RORγt degradation [122]. In line with the importance of RORγt ubiquitination, its deubiquitination by USP4 deubiquitinase results in RORγt stabilization and enhanced expression of Th17-associated genes [129]. Interestingly, in a different mode of regulation, DUBA, a deubiquitinase, stabilizes the ubiquitin ligase UBR5 which in turn promotes RORγt instability and suppresses Th17 differentiation [130]. In addition to the above (de)ubiquitinases, ubiquitin-specific protease USP17 can prevent polyubiquitination of human RORγt at Lys360 and stabilize RORγt. USP15 can deubiquitinate mouse RORγt at Lys446 to stimulate recruitment of coactivator NCoA1/SRC1 to IL-17A gene in promoting Th17 differentiation [131,132].

As one can expect, the major lysine acetyltransferase KAT3B/p300 interacts with RORγt and acetylates the receptor at several sites. One study demonstrated that acetylation in the DBD of mouse RORγt at Lys69, Lys81 and Lys 99 strongly impairs RORγt interaction with RORE and its induction of Th17 differentiation [121]. The study continued to show that deacetylase SIRT1

also interacts with the receptor and deacetylates its DBD, thus promoting the RORγt function in Th17 differentiation and its associated autoimmunity, particularly in the context of RORγt-expressing CD4+ T cells. However, a different study found that SIRT1, when activated by pharmacological reagents such as resveratrol, metformin and SRT1720, interacts with and deacetylates STAT3 which results in STAT3 localization to the cytoplasm and suppression of Th17 induction [133]. Activation of STAT3 induces the expression of RORγt and thus is required for the initiation of Th17 transcription program [134]. Intriguingly, in syngeneic models of B16F10 melanoma and CT26 colon cancer, pharmacological activation of SIRT1 inhibits the tumor growth and Th17 polarization from CD4+ T cells isolated from mouse draining lymph nodes, tumor-infiltrating lymphocytes and circulating lymphocytes of metastatic colon cancer patients [133]. Given the lineage plasticity and distinct chromatin landscape of different immune cells, it is likely that the exact role of RORγt or RORγ PTMs in regulation of the receptor function will depend on the cellular status of the NR-expressing cells and their microenvironment.

In cancer cells, a recent study demonstrated that PDZ binding kinase (PBK, also known as TOPK) interacts with RORγ and stabilizes RORγ protein by inhibition of RORγ ubiquitination [93]. PBK is a serine/threonine kinase that relates to the dual specific MAPKK family and displays pro-tumorigenic function in several cancer types [135]. PBK is overexpressed in a number of malignancies including prostate cancer, breast cancer and pancreatic cancer. Overexpressed PBK promotes therapeutic resistance and metastasis. Interestingly, studies showed that PBK is a downstream target of RORγ and AR in prostate cancer cells [93,135]. Combined with the previous finding that RORγ controls AR gene, the data together support a model where RORγ, AR and PBK reciprocally regulates each other and constitutes interlocked feedforward loops in amplifying AR signaling in metastatic CRPC [93]. However, the role of PBK in regulation of RORγt in immune cells is currently unclear.

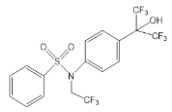
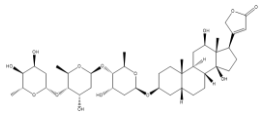
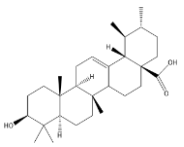
2.8 Therapeutics targeting RORγ and RORγt

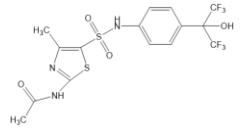
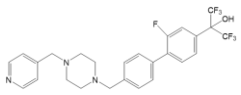
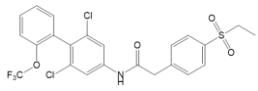
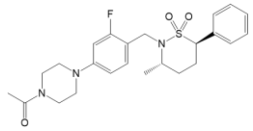
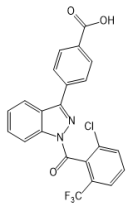
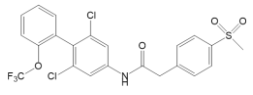
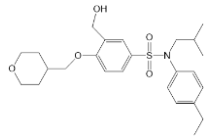
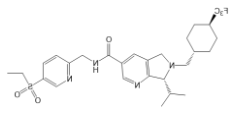
Synthetic ligands of RORγt identified in early years

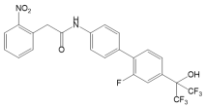
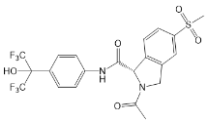
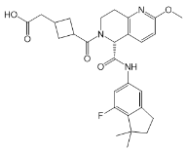
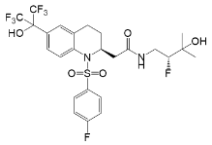
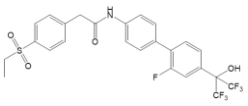
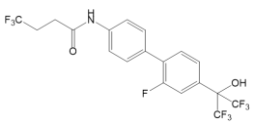
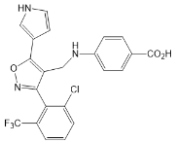
As members of the orphan NR subfamily, their initial gene identification and demonstration of potential functions in control of metabolism and immunity stirred strong interests of the field in searching for synthetic ligands in hope of developing effective agents for treatment of inflammatory diseases and metabolic disorders. The synthetic ligands identified early on are compound T0901317, its SR-series of derivatives and digoxin [136] (Table 2.2). The nonsteroidal benzenesulfonamide T0901317 was initially identified as a potent agonist of LXRs with strong activities in induction of fatty acid biosynthesis gene program in cell culture and mice [137]. T0901317 was later shown to be somewhat promiscuous in targeting other nuclear receptors including RORα and RORγ. It binds to the RORs with good affinity and significantly inhibits their activation of G6Pase, CYP7B1 and IL-17A in reporter assays [138]. The findings that RORγt plays crucial roles in promoting CD4+ T cell differentiation to Th17 and in Th17-associated autoimmune diseases (such as psoriasis, Crohn's disease and rheumatoid arthritis) prompted the search for its synthetic ligands with therapeutic values. In a screen of over 4800 compounds using a Drosophila cell-based reporter assay system, one study identified digoxin as a potent, RORγ-selective antagonist with EC50 of close to 2 μM [139]. Digoxin belongs to the cardiac glycoside family of drugs for treatment of atrial fibrillation and heart failure via inhibition of the cellular Na+/K+ ATPase. Interestingly, digoxin, not structurally related digoxigenin, can suppress IL-17A gene induction and Th17 cell differentiation. It displays a strong anti-inflammatory effect in a mouse model of Th17-mediated autoimmune disease, thus providing a prove-of-principle evidence that RORγt-targeting, small molecule therapeutics can be developed for many forms of inflammatory diseases [139]. Through optimization of T0901317, more selective compounds were identified [66]. SR1001 was identified as a first-in-a-class of synthetic inverse agonist/antagonist with

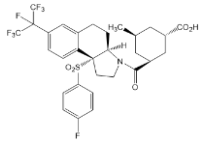
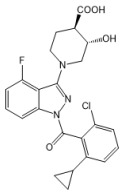
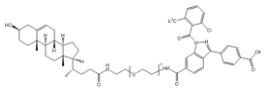
better selectivity to RORα and RORγ and potent activities in inhibition of Th17 cell differentiation and Th17-mediated multiple sclerosis in animal models [140]. SR1001 binds to the LBD and induces a conformational change with helix 12 re-positioning, which is linked to a switch of RORγ co-factor association from coactivator to corepressor. Through modification of the scaffold of SR1001, a study identified SR2211 as a RORγ-selective antagonist with an EC50 of 0.3 μM and more than 100-fold selectivity over RORα and LXRα in reporter gene assays [141]. SR2211 also displays potent activities in suppression of IL-17A gene expression and reduction of inflammation in animal models [141,142]. Differential hydrogen/deuterium exchange (HDX) mass spectrometry analysis demonstrated that SR2211 likely makes multiple direct contacts with the LBD of RORγ. Through structure-based optimization, other derivatives of T0901317 with improved RORγ-inhibitory biochemical and cellular activities were also identified [143] (Table 2.2).

Table 2.2 Synthetic ligands of RORγ

Name	Structure	year identified as RORγ ligand	target receptor	other targets	Ligand type	Clinical development				Ref.
						Disease	Phase	Dosing	current status	
T0901317		2010	LXR FXR PXR RORα RORγ	N/A	LXR, FXR, PXR: agonist RORα, RORγ: inverse agonist	N/A	N/A	N/A	N/A	[138,171-173]
Digoxin		2011	RORγ	Na^+/K^+-ATPase	RORγ: agonist (low concentration) inverse agonist (high concentration) Na+/K+-ATPase: inhibitor	Prostate cancer	Phase II	125 or 150mg orally daily for 24 weeks	Completed	[139,174]
						Metastatic breast cancer	Phase II	0.25mg digoxin once daily combined with capecitabine 650 mg/m^2 PO twice daily	Terminated	
Ursolic acid		2011	RORγ	various molecule	RORγ: inverse agonist	Prostate cancer	Early Phase I	150mg ursolic acid combined with 600mg curcumin twice a day to subjects who are scheduled to undergo radical prostatectomy	Not yet recruiting	[175]

SR1001		2011	RORα RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[140]
SR2211		2012	LXRα RORγ	N/A	LXR: weak agonist RORγ: Inverse agonist	N/A	N/A	N/A	N/A	[141]
GSK805		2014	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[101]
GNE-3500		2015	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[153]
MRL-871		2015	PPARγ RORγ	N/A	PPARγ: unclear RORγ: Inverse agonist	N/A	N/A	N/A	N/A	[157]
Compound 9a		2015	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[154]
GSK-2981278		2016	RORγ	N/A	Inverse agonist	Psoriasis	Phase I Phase II	4% ointment twice daily for 8 weeks	Completed	[151]
VTP-43742		2016	RORγ	N/A	Inverse agonist	Psoriasis	Phase I Phase II	Oral capsule at various dose once daily for 28 days	Completed	[144]

XY018		2016	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[90]
AZD-0284		2017	RORγ	N/A	Inverse agonist	Dose finding study	Phase I	Oral solution (2mg/mL and 15mg/mL) once or twice daily for 14 days (total volume 240mL)	Completed	[144]
						Plaque Psoriasis Vulgaris	Phase I	Oral solution 2.5 mg/mL, 100 mg twice daily for for 4 weeks	Terminated	
TAK-828F		2018	RORγ	N/A	Inverse agonist	Dose finding study	Phase I	Single oral dose between 0.1mg to 150mg	Completed	[155]
Compound 35		2018	RORγ PXR	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[145]
XY101		2019	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[162]
Compound 31		2019	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[162]
FM26		2020	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[159]

BMS-986251		2020	RORγ	N/A	Inverse agonist	Psoriasis	Phase I Phase II	Escalating oral dose	Terminated	[150]
N-(Indazol-3-yl)piperidine-4-carboxylic Acid		2020	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[158]
Bit-L-15		2021	RORγ	N/A	Inverse agonist	N/A	N/A	N/A	N/A	[161]

Inverse agonists/antagonists of RORγt identified for autoimmune diseases

The excellent potency and different structural features of the few synthetic ligands identified by the early studies (Table 2.2) strongly suggest that RORγ is highly amenable to modulation by structurally distinct ligands. In the years following the pioneer work, several pharmaceutical industry and academic laboratories reported their discoveries of a large number of RORγt inverse agonists with diverse chemical structures [144] (Table 2.2). The high throughput screening of a compound library was carried out either in a cell-based luciferase reporter system or in an in vitro binding assay. The reporter system was used to identify compounds that strongly suppress the transcriptional activation activity of RORγ LBD. In addition to IL-17 gene promoter-driven reporter, to facilitate the screening, the reporter could also be driven by fusion proteins of RORγ LBD and GAL4 DBD assembled at multimerized GAL4 binding sites in culture of Jurkat or other cell lines [145]. The in vitro, TR-FRET based binding screen identified compounds that could disrupt the association of recombinant RORγ LBD protein with a co-activator peptide [146-148]. Following the screening, hit compounds were subject to the in vitro binding assay and/or cell-based reporter assay to identify compounds with excellent

profiles in binding affinity and activation inhibition and with strong selectivity to RORγ when assayed against the other NR members. Their activity in suppression of IL-17A gene expression was also measured with CD4+ T cells. Experiments of structure-activity relationship (SAR) were usually performed to obtain candidate compounds with optimized profiles of solubility, PK and PD before they were tested in animal models of autoimmune disorders. In most of the published studies, co-crystal structures of the identified compounds with RORγ LBD were also presented. Based on the notion that cholesterol metabolites could be endogenous ligands of RORγ, a library screening was performed for compounds that displace radio-labeled 25-hydroxycholesterol from RORγ LBD and led to identification of tertiary sulfonamides as inverse agonists [149].

Structural analyses revealed that most of those ligands bind to the previously defined pocket of RORγ LBD (and thus are considered as orthosteric). Interestingly, some binds to an allosteric site. In a recent summary, the majority of the orthosteric ligands were categorized into four types based on their chemotypes, which include sulfonamides of cyclic amines, acyclic sulfonamides and sultam derivatives, aryl sulfonyl compounds, and amide/bis-amide derivatives [144] (Table 2.2). Representative compounds include bicyclic hexafluoroisopropyl alcohol sulfonamide or compound 35 from BMS[145] and their recent tricyclic analog BMS-986251 [150], GSK-2981278 [151,152], GNE-3500 [153], compound 9a [154], GSK805 [101], VTP43742 [144], AZD-0284 [144], and TAK-828F [155]. Most of those compounds have been at phase I or phase II clinical trials for potential use in treating autoimmune diseases. Other RORγt inhibitors such as JTE-151, JTE-451, ARN-6039, PF-06763809, ABBV-157 and SAR-441169 have also been reported at clinical development for autoimmune diseases such as psoriasis [156]. Except for ABBV-157, SAR-441169 and AZD-0284, trials of the other compounds have ended after phase I or II for the autoimmune disorders largely due to their lack of expected efficacies.

In a binding-based screen followed by hit optimization for compounds that disrupt the interaction of RORγ and SRC1 coactivator, MRL-871 with indazole chemotype was identified and later found to bind to an alternative site of RORγ LBD [157]. The co-crystal structures revealed that MRL-871 binds to an allosteric pocket that is predominantly hydrophobic and formed by helices H3, H4, H11 and H12 where MRL-871 binding reorients H12 in a conformation that precludes the LBD binding by the coactivator peptide. Other biochemical and cellular assays demonstrated that MRL-871 possesses high potency and selectivity to RORγ and RORγt-dependent Th17 production of IL-17. Further SAR studies of MRL-871 led to the identification of N-(Indazol-3-yl)piperidine-4-carboxylic acid as a new lead allosteric inhibitor with attractive profiles of PK, metabolic stability, anti-Th17 differentiation potency and potentially clean off-targeting [158]. In an in silico pharmacophore screen, a recent study identified FM26 with isoxazole chemotype as another allosteric inhibitor of RORγ [159]. Interestingly, in a competitive TR-FRET coactivator peptide recruitment assay, increasing amount of cholesterol (as an allosteric ligand) reduced the IC50 value of FM26 and thus enhanced FM26 binding to the LBD. Such a cooperative binding mode was also observed between cholesterol precursors such as desmosterol and its metabolites 20a-hydroxycholesterol and 25-hydroxycholesterol, and the other allosteric ligand MRL-871 and FM26 [160]. The discovery of a RORγ allosteric site significantly expands the strategy in searching for ligands with distinct profiles because amino acid sequences constituting the allosteric pocket are less conserved in the different RORs. Also, the existence of two pockets in one LBD would allow discovery of dual targeting drugs. In a recent prove-of-principle study, Bit-L-15 was identified as a bitopic ligand of RORγ with cholesterol and MRL-871 linked by a polyethylene glycol (PEG) linker. Bit-L-15 displayed improved potency in disrupting coactivator recruitment and improved NR selectivity over its orthosteric and allosteric parental compounds [161].

Inhibitors/antagonists of RORγ with strong anti-cancer activities

In contrast to the large number of compounds identified for inhibition of RORγt function in Th17 cell differentiation and autoimmune diseases, very few chemical structures have been reported that target RORγ in cancer cells and tumors. The finding that RORγ plays a crucial role in prostate cancer [90] prompted identification of RORγ inhibitors with high potency in cancer cells. One initial attempt was combining the structural features of SR2211 and one of the GSK compounds which displayed excellent activities in suppressing RORγt function in Th17 cells [101,142]. By combining the hexafluoropropan-2-ol group of SR2211 and the amide group of the GSK compound and by structure-based optimization, the study identified XY018/compound 23 and compound 31 with amide linker to possess a potent inverse agonist with a strong activity in decreasing AR full-length and variant protein expression and AR signaling in the prostate cancer cells [90,93,162]. Those compounds, like SR2211, displayed excellent potency in inhibition of the growth of prostate and breast cancer xenograft tumors [90,93,95,162]. Additional SAR analysis with the luciferase reporter and thermal shift assay (TSA) identified XY101/compound 27 with ethyl sulfonyl connected to a benzyl group at the side of amide linker as another potent inhibitor of RORγ with good selectivity over the other NRs and excellent activities in disruption of RORγ interaction with the coactivator peptide. Cocrystal structure with XY101 revealed several interactions in the form of H-bonds and π-π interactions between the amino acids of the LBD mainly in the region of H3 to H6 and the compound linker amide, the middle phenyl ring and the ethyl sulfone. In addition, there appears a potential disruption by the hexafluoisopropanol group of the so called His-Tyr lock, namely hydrogen bond between His479 in helix 11 and Tyr502 in helix 12 [162]. This later engagement with the LBD has also been observed in many of the other inverse agonists [144,145,163,164]. Intriguingly, a recent study reported that elaiphylin, an antibiotic isolated from a marine species of Streptomyces, can directly bind to RORγ LBD and exert strong inhibition of its activation

function including its target genes in prostate cancer cells. It also displayed strong activities in inhibition of xenograft tumor growth [165].

2.9 Conclusion and future perspectives

The last decade has witnessed a tremendous progress in elucidation of aberrant cholesterol metabolism and homeostasis in cancer development and progression and identification of the underlying major drivers and regulators. The statistically positive association between the use of statins among cancer patients and better clinical outcomes of their malignant disease and the excellent safety profile of statins as drugs for cardiovascular diseases prompted a large number of preclinical and clinical studies with the goal to re-purpose statins for cancer therapy. Despite the promising results from preclinical studies, statins either alone or in combination with other cancer therapies have not shown any significant and consistent benefits to cancer patients in the trials. Many factors may contribute to the lack of success which include the prominent feedback up-regulation of cholesterol biosynthesis program in treated tumors, the lack of reliable biomarkers to patient selection, and the realization that effective cancer treatment by statins demands doses higher than what safe use would allow [5]. Because of the pivotal regulatory role of SREBP2 in cholesterol metabolism, therapeutic strategies targeting SREBP2 were also proposed and examined in preclinical setting [6]. However, with its crucial role in both normal and cancer cells, safe targeting of SREBP2 for cancer therapy is likely to be challenging.

NR family member RORγ and its immune cell isoform RORγt, a well-known player in circadian rhythm, metabolism, Th17 differentiation and autoimmune disorders, emerged recently as a major regulator of cholesterol biosynthesis in a subtype of breast cancer [95]. Like the established master regulator SREBP2, RORγ directly activates almost the entire cholesterol biosynthesis program in the cancer cells. Unlike SREBP2 which exerts its function in most of the normal and cancerous tissues, this newly defined RORγ function appears to be restricted to

certain types of cancer cells and tumors, which suggest that other tumor subtype-specific regulators of cholesterol metabolism are likely in play and await to be identified. Although both SREBP2 and RORγ can be regulated by products of cholesterol pathways, the mode of regulation is vastly different for these two TFs. While SREBP2 is usually subject to a negative feedback regulation by increased level of cholesterol, based on the results from the immune cells, RORγ is likely further activated by increased level of certain cholesterol precursors which would constitute a positive feedforward loop in the biosynthesis pathway (Figure 2.3). However, future investigations are needed to establish the loop in the immune cells and tumors. Such a positive loop, if confirmed, would beg a question about its biological importance. It is well established that most solid tumors exhibit a hyperactive activity in cholesterol biosynthesis and metabolism and possess a significantly higher content of cholesterol than normal tissues. One possibility is that regulators such as RORγ are needed to sustain the relatively high metabolic flux and cellular content of cholesterol in tumors where SREBP2 maintains the baseline of the metabolic activity as it does in normal tissues. It is also possible that the positive feedforward loop may serve as a fail-safe mechanism for cancer cells to ensure a constant supply of not only cholesterol but also other cholesterol metabolites that are needed for the high rate of cell proliferation and hyperactive cell membrane-engaged signaling. However, given the fact that excess cholesterol is toxic to the cells, it is conceivable that the RORγ-mediated positive loop is also subject to attenuation. One source of the possible attenuation might be specific cholesterol metabolites such as certain oxysterols that can act as an antagonist ligand of RORγ.

Compared to SREBP2, RORγ is a more attractive therapeutic target. One advantage over SREBP2 is that the LBD of RORγ intrinsically binds small molecules with excellent affinity and selectivity and thus permits effective targeting by synthetic small molecule ligands. The recent finding that RORγ possesses both orthosteric and allosteric sites for small molecules

increases the likelihood of identification of RORγ-targeting therapeutics with the most desirable potency and selectivity. The fact that RORγ controls the cholesterol pathway in a subtype of cancer supports the notion that therapeutics targeting the receptor could feature cancer subtype-specific response and better safety than therapeutics targeting SREBP2 which plays an essential role in both normal and tumor tissues. Nonetheless, a number of future investigations are warranted, which include identification of cholesterol intermediates and metabolites in specific tumor subtypes that modulate RORγ function, delineation of major oncogenic pathways that impinge on RORγ, and development of novel small-molecule inhibitors that preferentially target the unique function of RORγ in control of tumor metabolism. Moreover, studies on biomarkers or gene signatures of RORγ in tumor subtypes can facilitate rational design of combinatorial strategies with other drugs such as statins for more effective treatment of advanced cancers.

2.10 Acknowledgement

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Chapter 3

Nuclear receptor RORγ inverse agonists/antagonists display tissue- and gene-context selectivity through distinct activities in altering chromatin accessibility and master regulator SREBP2 occupancy

3.1 Abstract

The nuclear receptor RORγ is a major driver of autoimmune diseases and certain types of cancer due to its aberrant function in T helper 17 (Th17) cell differentiation and tumor cholesterol metabolism, respectively. Compound screening using the classic receptor-coactivator interaction perturbation scheme led to identification of many small-molecule modulators of RORγ(t). We report here that inverse agonists/antagonists of RORγ such as VTP-43742 derivative VTP-23 and TAK828F, which can potently inhibit the inflammatory gene program in Th17 cells, unexpectedly lack high potency in inhibiting the growth of TNBC tumor cells. In contrast, antagonists such as XY018 and GSK805 that strongly suppress tumor cell growth and survival display only modest activities in reducing Th17-related cytokine expression. Unexpectedly, we found that VTP-23 significantly induces the cholesterol biosynthesis program in TNBC cells. Our further mechanistic analyses revealed that the VTP inhibitor enhances the local chromatin accessibility, H3K27ac mark and the cholesterol master regulator SREBP2 recruitment at the RORγ binding sites whereas XY018 exerts the opposite activities, despite their similar effects on circadian rhythm program. Similar distinctions between TAK828F and SR2211 in their effects on local chromatin structure at *Il17* genes were also observed. Together, our study shows for the first-time that structurally distinct RORγ antagonists possess different or even contrasting activities in tissue/cell-specific manner. Our findings also highlight that the activities at natural chromatin are key determinants of RORγ modulators' tissue selectivity.

3.2 Introduction

Nuclear receptors (NRs) comprise a superfamily of structurally conserved, ligand-regulated transcription factors, serving as receptors for steroid hormones and derivatives of lipids and fatty acids [1, 2]. NRs share a common structure including a central DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD). Ligand binding induces a conformational change in the receptor, which results in its association with other co-regulatory proteins and regulation of gene expression. Notably, NR function is diverse and context-specific. For instance, glucocorticoid binding to glucocorticoid receptor (GR) can induce the death of thymocytes and osteoblasts or promote cell survival in liver and heart [3]. Dysfunction of NR signaling can lead to proliferative, reproductive and metabolic diseases such as cancer, infertility, obesity and diabetes. Thus, the NR superfamily is one of the primary classes of therapeutic drug targets for human diseases [4, 5]. For example, dexamethasone and many other potent agonists of GR are used in treatment of autoimmune and inflammatory diseases. Tamoxifen, a selective estrogen receptor modulator (SERM) acting as an antagonist in mammary tumor cells but as a partial agonist in the uterus, has been widely used for treatment of estrogen receptor (ER)-positive breast cancer [6].

Recent studies have revealed important roles played by retinoic acid receptor-related orphan receptor-gamma (RORγ) in control of immunity, circadian rhythm, metabolism and certain type of cancer [7-9]. RORγ is encoded by RORC/NR1F3 gene and belongs to the ROR subfamily of NRs. RORγ protein has two different isoforms, RORγ and RORγt, which differ only in the N-terminus but display distinct expression patterns [7]. RORγ is widely expressed in many tissues, including liver, adipose, skeletal muscle, and kidney, while RORγt is exclusively highly expressed in thymus and other immune cells [8]. Although RORγ was initially characterized as an orphan receptor, recent studies strongly suggest that certain intermediates of cholesterol biosynthesis and specific metabolites of cholesterol and bile acids

are its endogenous ligands [7, 8]. Similar to GR [3], RORγ also displays tissue-specific functions. RORγ is found to regulate circadian rhythm gene expressions in liver [10]. RORγ knock-out mice are protected from hyperglycemia and insulin resistance in obesity [11]. RORγt is essential for differentiation of naïve $CD4^+$ T cells to T helper 17 (Th17) cells that produce interleukin 17 (IL-17) cytokines. Aberrant functions of RORγt result in overproduction of IL-17 cytokines in Th17 and other immune cells which can lead to development of different autoimmune diseases. Therefore, RORγt is a promising target for the autoimmune diseases [8, 12]. Studies from us and others demonstrated that RORγ in tumor cells can promote tumor growth and metastasis in castration-resistant prostate cancer, triple-negative breast cancer (TNBC) and pancreatic adenocarcinoma [13-18]. Recent studies also demonstrated that RORγ is a novel master regulator of tumor cholesterol biosynthesis in TNBC [18]. Thus, targeting RORγ is a promising strategy for effective treatment of specific types of cancer.

An increasing number of synthetic and natural small molecules with distinct chemical structures are being developed targeting RORγ or RORγt with the majority of them displaying inverse agonist (if RORγ(t) in the cell is considered unliganded) or antagonist (if RORγ(t) in the cell is bound with an agonist ligand) activities [7, 19-21]. One common strategy used in identification of those synthetic ligands is based on their activities in disrupting the interaction between the receptor LBD and a LXXLL motif-containing peptide derived from an NCoA/SRC coactivator. Among the inverse agonists identified (Fig. 3.1A), TAK828F [22], VTP-43742 [23] and its derivative VTP-23 [19] have been at clinical trials for treatment of autoimmune diseases. Others such as GSK805 [24], SR2211 [25] and XY018 [13] display potent activities in inhibition of tumor growth and metastasis [13-18]. However, there has not been any side-by-side, direct comparison between those RORγ modulators in a given platform or system. Through measuring their activities in the immune and cancer cell models, we found that those RORγ modulators display a large difference in suppression of RORγ function in the different

cells. Unexpectedly, our study also revealed that compounds that are highly potent in blocking RORγt in the immune cells appear to display agonist activities in the cancer cells. Using epigenetic approaches, our study showed that differences in their cellular activities are linked to their distinct ability in altering local chromatin structure and recruitment of the cholesterol master regulator SREBP2 at the natural RORγ targets. Together, our study demonstrated for the first-time that structurally distinct small-molecule RORγ modulators possess tissue/cell-selective activities in perturbing the function of RORγ or RORγt through their distinct actions at the natural chromatin site. We anticipate that our findings will entice future development of truly tissue-selective RORγ modulators.

3.3 Materials and Methods

Cell culture

HCC70 cell was cultured in RPMI1640 supplemented with 10% FBS. MDA-MB-468 and EL4 cells were cultured in DMEM supplemented with 10% FBS. Cells were grown at 37 °C in 5% CO2 incubators. Cells were obtained from ATCC and were regularly tested being negative for mycoplasma.

Chemicals

Sources for chemicals are as follows: TAK828F, VTP compound 23 (VTP-23) [19], XY018 and GSK805 (purity > 99%) were synthesized by WuXi AppTec. Other chemicals (purity > 97%) are from Sigma and Cayman unless indicated otherwise.

Naïve CD4+ T-cell isolation and Th17 differentiation

CD4+CD62L+ naïve CD4+ T cells were purified from C57BL/6 mice (Envigo) using EasySep™ Mouse CD4+CD62L+ T Cell Isolation Kit (Cat. 18765, Stemcell). Naïve CD4+ cells were cultured in anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml) antibody pre-coated 96-well plates and treated with different RORγ antagonists or vehicle. For Th17 cell differentiation, cultures were supplied with IL-6 (20 ng/ml) and TGF-β1 (1 ng/ml). Forty-eight hours later,

cultures were further supplied with IL-23 (10 ng/ml) and IL-1β (10ng/mL). After 96 hours of compound incubation, cells were treated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Sigma) and ionomycin (1 μg/mL) for 4 hours before collected for further experiments.

Cell viability, proliferation and colony formation

For cell viability, cells were seeded in 96-well plates at 1000-2000 cells per well in a total volume of 100 μL of media. After 4 days of incubation of compounds, Cell-Titer Glo reagents (Promega) were added, and luminescence was measured on GLOMAX microplate luminometer (Promega) or Varioskan™ LUX multimode microplate reader (Thermo Scientific), according to the manufacturer's instructions. All experimental points were set up as triplicate as biological replication, and the entire experiments were repeated three times. The data are presented as percentage of viable cells with vehicle-treated cells set as 100. The estimated in vitro IC50 values were calculated by using GraphPad Prism 9 software. For cell proliferation, cells were seeded in 6-well plates at 2 X 105 per well and treated as indicated. Total viable cell numbers were counted using Countess™ II Automated Cell Counter (Invitrogen). For colony formation assay, 500 cells were seeded in a well of 6-well plate and cultured for 21 days with the medium changing every 5 days. When the cell clone grew visible, the medium was removed, and the cells were fixed with 10% formalin for 10 mins. The plated were washed with PBS for two times, and cell colonies were stained with 0.2% crystal violet (in 10% formalin) for 30 mins. The above assays were performed in duplicates, and the entire experiments were repeated three times.

qRT- PCR and western blotting analysis

Total RNA was isolated from cells using TRIzol™ Reagent (Cat. 15596018, Invitrogen). The cDNA was prepared using qScript™ cDNA SuperMix (Cat. 95048-100, QuantaBio). Quantitative PCR were performed as previously described with modification [26]. Briefly, cDNAs were mixed with SYBR Green qPCR master mix (B21203, Bimake) and gene specific

primers. The PCR were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). The fluorescent values were collected, and fold difference was calculated. GAPDH was used as the internal reference to normalize the relative level of each transcript. The experiments were performed at least three times. Primers are listed in Table 3.1.

Cell lysates were analyzed by immunoblotting with antibodies specifically recognizing RORγ and GAPDH. The antibodies used are shown in Table 3.2.

RNA-seq and bioinformatics analysis

MDA-MB-468 cells were treated as indicated before RNA extraction. RNA-seq libraries from 1 μg total RNA were prepared and validated as previously described. Sequencing was performed on an Illumina HiSeq 2000 Sequencer at BGI Tech (Hong Kong). The FASTQ-formatted sequence data were analyzed using a standard BWA-Bowtie-Cufflinks workflow. Briefly, sequence reads were aligned to the reference human genome assembly (hg19) with BWA and Bowtie software. Subsequently, the Cufflinks package [27]. was applied for transcript assembly and quantification gene expression. To avoid spurious fold levels due to low expression values, only subsets of genes that have expression value of RPKM (reads per kilobase per million mapped reads) or FPKM (fragments per kilobase of exon model per million mapped reads) above 1 for either the vehicle treated cell, or the compound treated cells are included. GSEA was performed using the Java desktop software (http://www.broadinstitute.org/gsea) as described previously [28]. Genes were ranked according to the shrunken limma log2 fold changes and the GSEA tool was used in 'pre-ranked' mode with all default parameters. Previous reported cholesterol biosynthesis genes [29] were used in the GSEA analysis.

Reporter constructs and reporter-gene assay

Transient transfection and reporter-gene assays were performed as previously described, with the following modifications. For RORE reporter-gene assays 7 X RORE and 4 X AR-RORE-

tk-luc was constructed as previously described [13]. Cells were co-transfected with pLX304-RORγ or empty vector and indicated RORE reporter plasmid using lipofectamine 3000 (Cat. L3000015, Invitrogen). Renila plasmid was co-transfected for normalization. After 12 hours of incubation, cells were treated with vehicle or RORγ inhibitors as indicated for another 24 hours. The luciferase activity was analyzed using Dual-Glo Luciferase Assay System (Promega) on GLOMAX microplate luminometer (Promega) or Varioskan™ LUX multimode microplate reader (Thermo Scientific), according to the manufacturer's instructions. All transfections were performed in triplicate, and each experiment was repeated at least three times.

ChIP-qPCR analysis

ChIP-qPCR analysis was performed as described previously [18]. The antibodies used for the ChIP assay are SREBP2 (Cayman, 10007663); H3K27ac (Diagenode, C15410196) and IgG (Cell signaling technology, 2729S). ChIPs were performed with each experimental point in triplicate, and each experiment was repeated three times. The primers are shown in Table 3.1.

Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)

Samples were prepared as described previously [30]. Paired-end 100bp sequences were generated from samples on an NovaSeq platform at BGI Tech (Hong Kong). Fastq files from ATAC-seq were processed by the pipeline on Github (https://github.com/ENCODE-DCC/atac-seq-pipeline). Briefly, sequencing tags were mapped against the murine genome (mm9) or Homo sapiens (human) reference genome (hg19) by using Bowtie 2.2.6. Mitochondrial and duplicated reads were filtered through SAMtools (v 1.2) and picard (v 2.1.1, https://www.broadinstitutegithubio/picard/). After filtering and deduping, uniquely mapped tags were used for peak calling by model-based analysis (MACS; 2.1.0) to identify regions of enrichment over background. Peaks that overlapped blacklisted regions were removed. After normalization, genome-wide signal-coverage tracks from raw-read alignment files were built by MACS2, UCSC tools (bedGraphToBigWig/bedClip;

http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/), and bedTools (https://github.com/arq5x/bedtools2). The ATAC-seq signal visualization at enriched genomic regions (avgprofile and heatmap) was achieved by using deepTools (https://deeptools.readthedocs.io/en/develop/index.html). The resulting sets of ATAC-seq peaks were inferred as high confidence Tn5 hypersensitive site (THSS) regions. The annotation of THSS regions to genomic features was performed using the HOMER suite tool annotatePeaks (http://homer.ucsd.edu/homer/index.html). Each of the sites was assigned to the nearest gene. Further annotation information includes whether a peak is in the TSS (transcription start site, from −1 kb to + 100 bp), TTS (transcription termination site, from −100 bp to + 1 kb), Exon (Coding), 5′ UTR Exon, 3′ UTR Exon, Intronic, or Intergenic.

Statistical analysis

Cell culture-based experiments were performed three times or more, with assay points triplicated. The data are presented as mean values ± SD. Statistical analyses were performed by GraphPad Prism software 9.

3.4 Results

3.4.1 RORγ antagonists with distinct structures display different potency in inhibition of RORγ-dependent transactivation function

Small-molecule modulators of RORγ or RORγt such as TAK828F [22], VTP-43742 and its improved analog VTP-23 [19], GSK805 [24] and XY018 [13] were previously developed by different laboratories with potent activities in models of autoimmune and cancer (Fig. 3.1A) (given the high likelihood that RORγ or RORγt in the cells or tissues is bound with endogenous agonistic ligands such as cholesterol biosynthesis intermediates or metabolites, hereafter we will describe them as antagonists or modulators). To directly compare their activities, we first performed luciferase reporter assays to measure their activities in antagonizing the transactivation of RORγ. Interestingly, in HEK293T cell, although all compounds diminished

the 7X-RORE and 4X-AR-RORE dependent activation in a concentration dependent manner, the inhibitory activities of those compounds differed markedly (Fig. 3.1B). Specifically, at 100 nM, TAK828F and VTP-23 showed more than 50% inhibition of RORγ-dependent transactivation at 7X-RORE and 4X-AR-RORE luciferase reporters. In comparison, it takes 1 μM of SR2211 or 5 μM of XY018 to elicit a similar percentage of inhibition. Similarly, over 10-fold difference in the inhibition potency among the antagonists were observed in a TNBC cell model (Fig. 3.1C). These data suggest that those RORγ antagonists with different structures display remarkable differences in inhibition of RORγ-dependent transactivation function in the reporter gene assays.

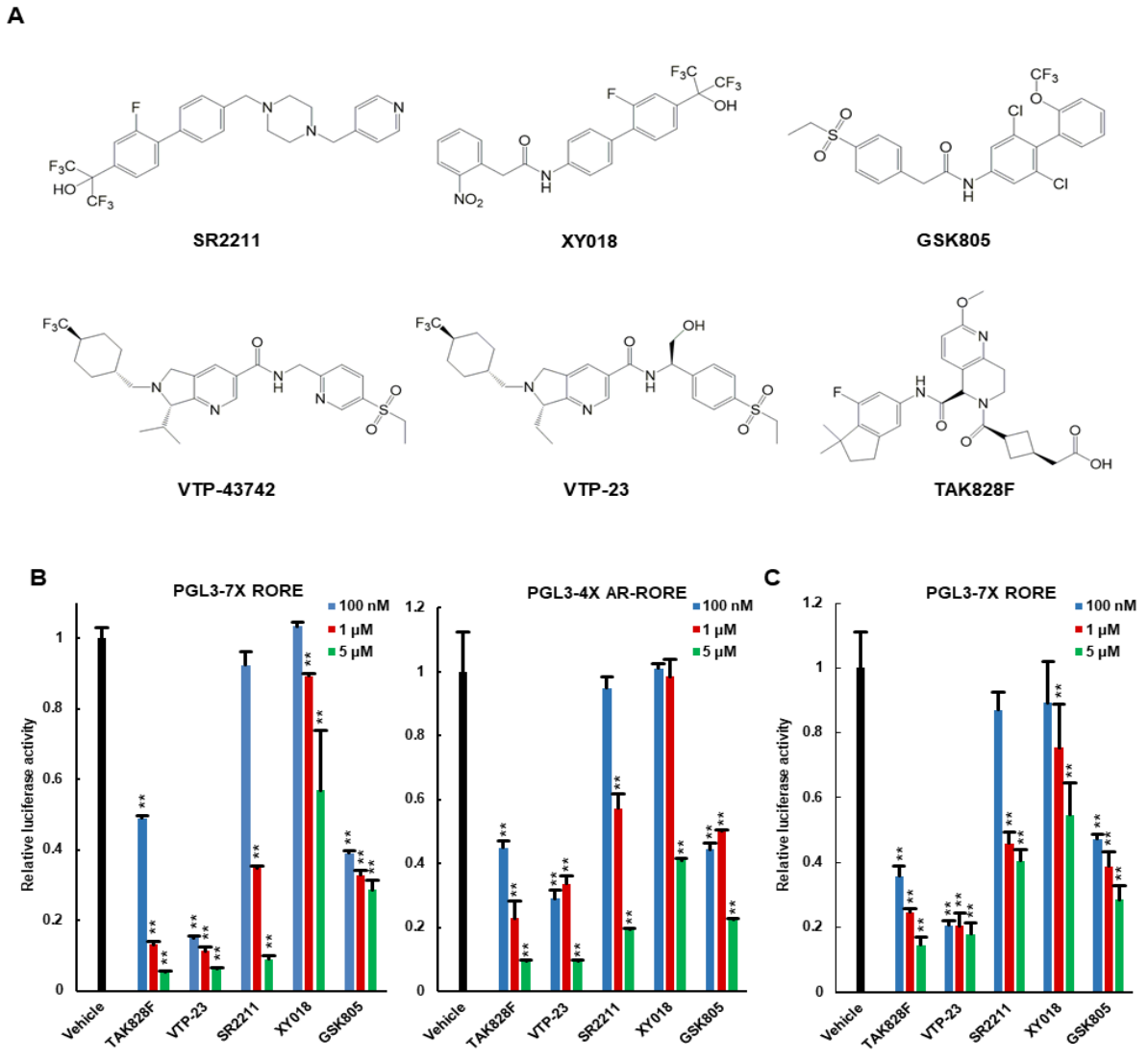


Figure 3.1. RORγ antagonists with distinct structures display different inhibitory activities at RORγ-dependent reporters

A. Chemical structures of five RORγ inhibitors: SR2211, XY018, GSK805, VTP-43742, VTP-23 and TAK828F. **B.** 7X-RORE and AR-RORE luciferase reporter activity changes by treatment of different concentrations of RORγ inhibitors in 293T cells for 24 hours. Normalized luciferase activity from cells treated with vehicle and transfected with RORγ-expressing plasmid were set as 1. Data are shown as mean ± SD. n=3. **C.** 7X-RORE luciferase reporter activity changes by treatment of different concentrations of RORγ inhibitors in MDA-MB-468 cells for 24 hours. Normalized luciferase activity from cells treated with vehicle and transfected with RORγ-expressing plasmid were set as 1. Data are shown as mean ± SD. n=3. Student's *t*-test. ** $p < 0.01$.

3.4.2 The different RORγ antagonists display marked difference in their potency of inhibition of Il17 gene induction in Th17 cells

RORγt plays a crucial role in induction of IL-17 cytokines in Th17 cells. We thus examined whether those RORγ or RORγt antagonists also display different activities in suppressing RORγt-dependent cytokine production in mouse Th17 cells. Naïve mouse CD4+ T cells isolated from C57BL/6 mice spleens were incubated with different RORγ antagonists under Th17 differentiation condition. As expected, all five compounds examined reduced both *Il17a* and *Il17f* expression in a concentration-dependent manner (Fig. 3.2A and B). However, although all of them inhibited 90% of *Il17a* and *Il17f* expression at a high concentration (1 μM) (Fig. 3.2C and D), TAK828F, VTP-23 and GSK805 showed significantly greater inhibitory activities at lower concentrations (10 and 100 nM), compared with the other RORγ antagonists SR2211 and XY018 (Fig. 3.2A and B). Specifically, 100 nM of TAK828F and VTP-23 exhibited more than 90% *Il17a* inhibition, whereas the same concentration of XY018 and SR2211 only inhibited 30% and 50% of *Il17a* mRNA expression, respectively. These results show that the RORγ antagonists examined display significant different potency in inhibiting

RORγ-mediated *Il17* gene expression in mouse Th17 cell, with the potency difference similar to that observed in the above luciferase reporter gene assays.

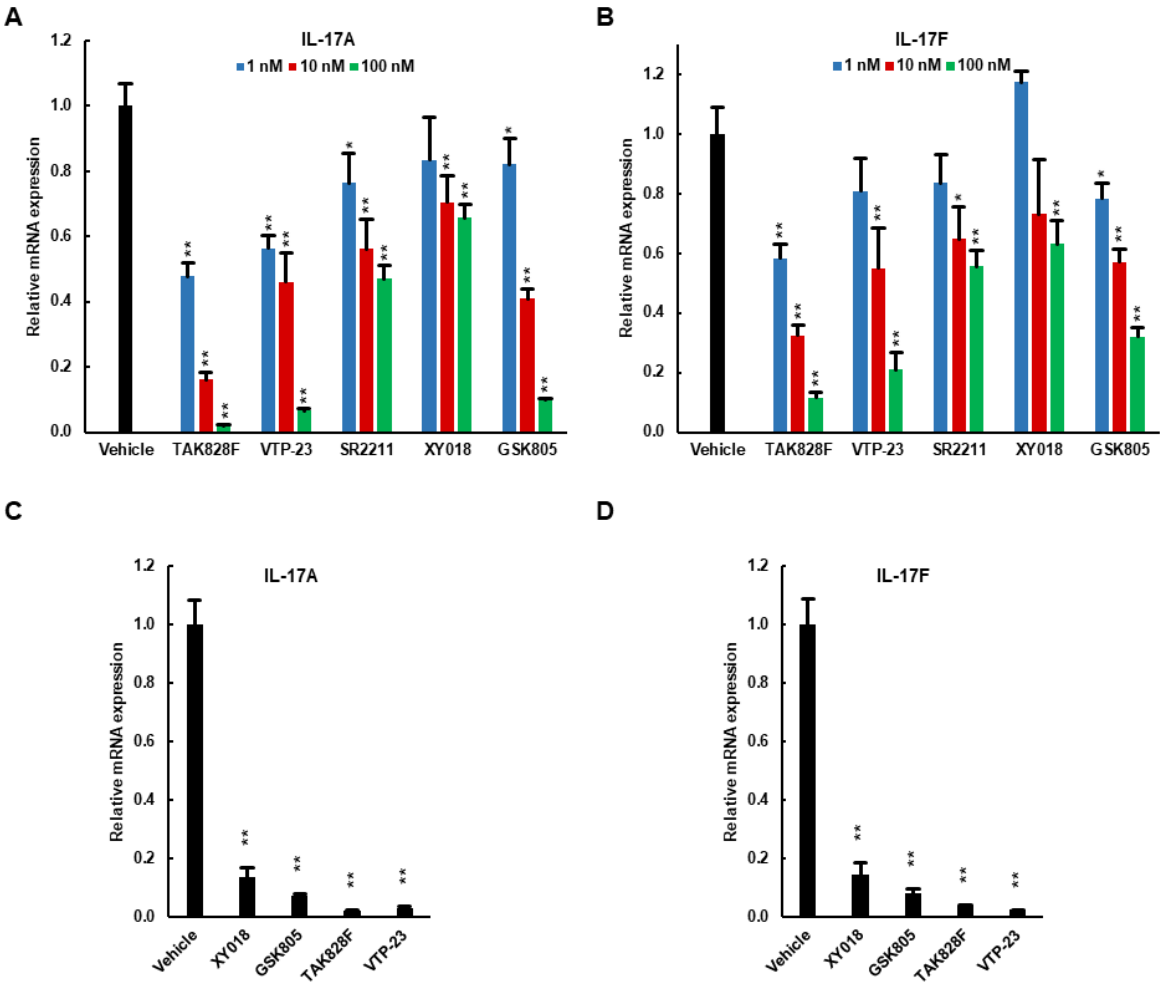


Figure 3.2 RORγ inhibitors exhibit different potencies in suppressing IL-17 cytokine production in mouse Th17 cells

A, B. Naïve CD4+ T cells were isolated from C57BL/6 mice and activated under Th17-cell-polarizing conditions in the presence of different RORγ inhibitors at indicated concentrations (1 nM, 10 nM and 100 nM). After 4 days, *Il17a* **(A)** and *Il17f* **(B)** expressions were measured by qRT-PCR. **C, D.** Naïve CD4+T cells were isolated and activated as in **(A)** in the presence of 1 μM of different RORγ inhibitors. After 4 days, *Il17a* **(C)** and *Il17f* **(D)** expressions were measured by qRT-PCR. Data are shown as mean ± SD. n=3. Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

3.4.3 RORγ antagonists display opposing effects on cholesterol biosynthesis program but similar effects on circadian rhythm program in the same cancer cells

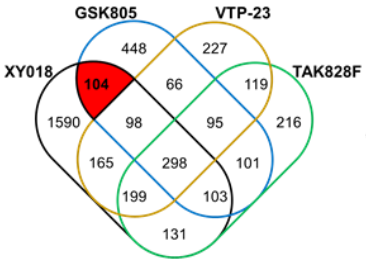
In our previous study, we demonstrated that RORγ is a novel master regulator for tumor cholesterol biosynthesis in TNBC. Pharmacological and genetic inhibition of RORγ reduces tumor cholesterol level and synthesis rate, leading to inhibition of the tumor growth [18].

To investigate whether the different compounds also display distinct activities in RORγ control of TNBC cell cholesterol biosynthesis program, we performed RNA-seq analysis of MDA-MB-468 TNBC cells treated with the RORγ antagonists. Gene ontology (GO) analysis of the 104 transcripts downregulated by both XY018 (2.5 μM) and GSK805 (2.5 μM), but not TAK828F (10 μM) or VTP-23 (10 μM), revealed that genes involved in cholesterol biosynthesis pathway were among the most highly enriched (Fig. 3.3A and B). On the other hand, transcripts that were downregulated only by TAK828F and VTP-23 were not involved in cholesterol biosynthesis program (Fig. 3.3C and D). Further examination by gene-set enrichment analysis (GSEA) also indicated clearly that hallmarks of cholesterol-biosynthesis program were significantly altered by both XY018 and GSK805 at a relatively low concentration (2.5 μM). However, TAK828F or VTP-23 treatment at either a low (2.5 μM) or a high (10 μM) concentration did not alter the cholesterol gene program (Fig. 3.3E). To examine whether the effects of RORγ antagonists on cholesterol biosynthesis program can be extended to other cells, we analyzed the gene expression in mouse CD4+ T cells undergoing differentiation to Th17 and treated by the antagonists and found that none of the antagonists displayed a significant alteration of mRNA expressions of the major cholesterol biosynthesis genes, including *Hmgcr*, *Hmgcs1*, *Sqle* and *Mvk* (Sup Fig.3.1). These data indicate that RORγ does not regulate cholesterol biosynthesis pathway in T cells.

Our further analysis revealed that the majority of genes involved in cholesterol-biosynthesis pathway, including two genes that encode the rate-limiting enzymes, *HMGCR* and *SQLE*, were

down-regulated in the TNBC cells by XY018 and GSK805 (Fig. 3.3F). To extend the analysis to different cells and different concentrations, we treated HCC70 TNBC cells with 5 μM of each compound and observed distinct effects by the different compounds similar to those observed in MDA-MB468 cells (Fig. 3.3G). Twenty-four hours treatment of GSK805 and XY018 resulted in a strong down-regulation of 19 out of the 21 cholesterol biosynthesis genes, including *HMGCS*, *HMGCR* and *SQLE*. In contrast, the expression of those genes remained largely unchanged when treated with TAK828F. Unexpectedly, a significant up-regulation by VTP-23 of several genes in the pathway (e.g. *HMGCS1, MVD*, *TM7SF2* and *DHCR7*) was observed in the TNBC cells (Fig. 3.3F and G). Circadian rhythm is another major program regulated by RORγ. Interestingly, VTP-23 significantly inhibited the expression of key circadian regulators such as *ARNTL*/*Bmal1*, *PER2*, *CRY1* and *FBXL3*, although its inhibition potency is lower than XY018 (Fig. 3.3H). To summarize, the above results show that GSK805 and XY018, but not TAK828F, strongly inhibit cholesterol biosynthesis gene expression whereas VTP-23 induces the same gene program. On the other hand, both XY018 and VTP-23 inhibit circadian rhythm gene expressions in the same TNBC cells. Therefore, the results strongly suggest that different RORγ antagonists possess distinct activity in modulation of RORγ functions in a cell- and gene program-specific manner.

A



B

GO analysis of genes down-regulated by XY018 and GSK805 only (104 genes)

Description	Log P
cholesterol biosynthetic process	-7.5
isoprenoid biosynthetic process	-2.7
sterol biosynthetic process	-1.3

C

GO analysis of genes down-regulated by VTP-23 and TAK828F only (119 genes)

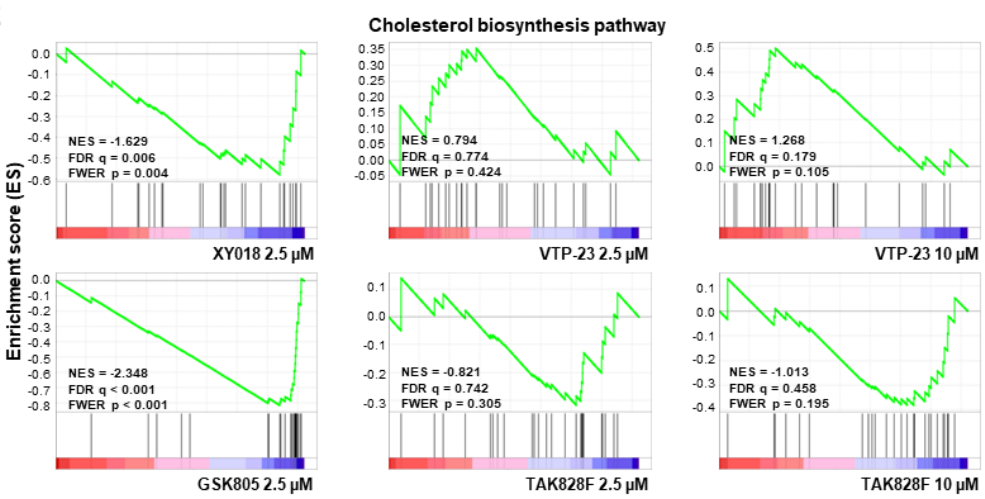
Description	Log P
transport	-2.5
negative regulation of angiogenesis	-2.3
chemical synaptic transmission	-2.0

D

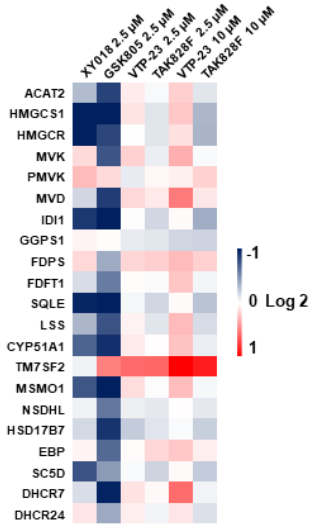
GO analysis of genes down-regulated by all four compounds (298 genes)

Description	Log P
G-protein coupled receptor signaling pathway	-3.6
chemokine-mediated signaling pathway	-3.4
immune response	-2.3
nucleosome assembly	-2.2
inflammatory response	-2.2

E



F



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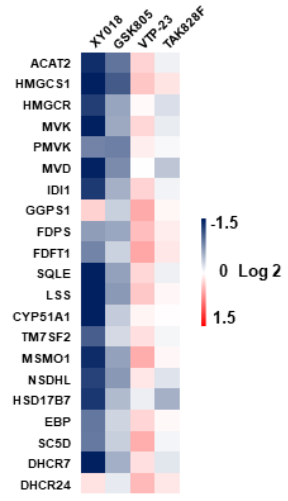

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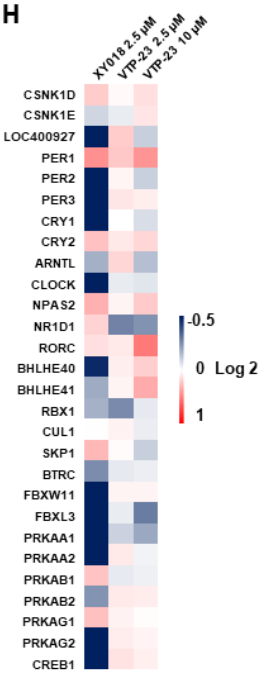
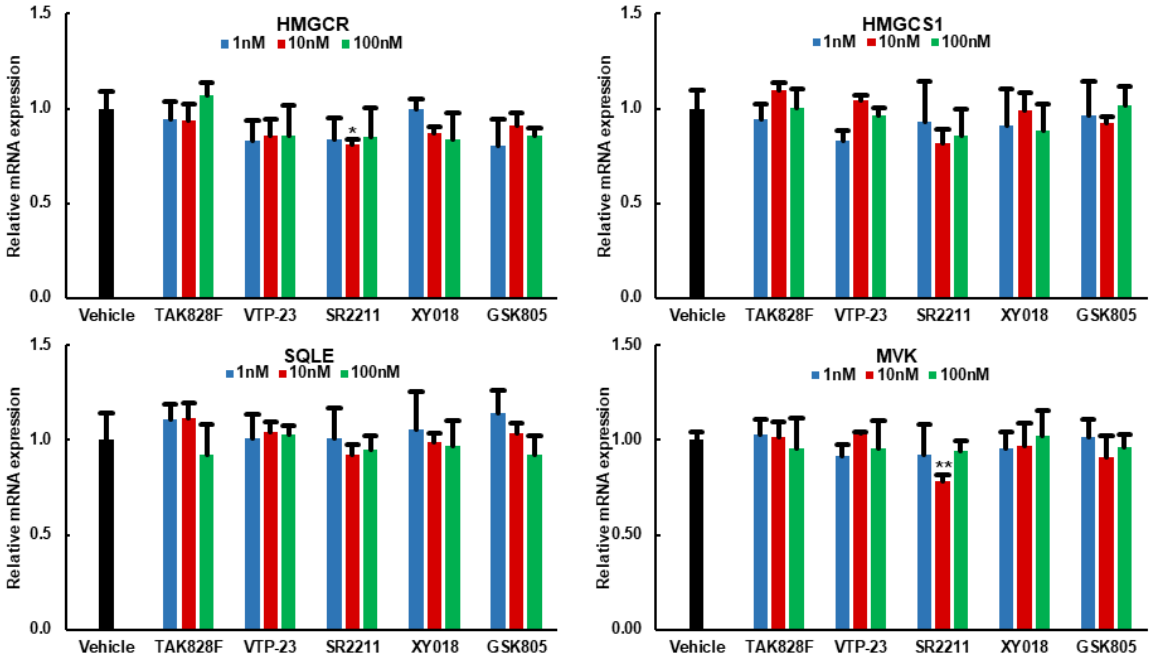


Figure 3.3 GSK805 and XY018, but not TAK828F and VTP-23, strongly inhibit cholesterol biosynthesis pathway genes in TNBC cells

A. Venn diagram of the number of genes with expression significantly (1.5-fold) reduced, which is detected by RNA-seq of MDA-MB-468 cell treated with XY018 (2.5 μM), GSK805 (2.5 μM), VTP-23 (10 μM) or TAK828F (10 μM) for 24 hours. **B-D. (B)** Gene ontology analysis of 104 genes downregulated by both XY018 (2.5 μM) and GSK805 (2.5 μM), but not TAK828F (10 μM) or VTP-23 (10 μM) as shown in **(A)**. **(C)** Gene ontology analysis of 119 genes downregulated by both TAK828F (10 μM) VTP-23 (10 μM), but not XY018 (2.5 μM) or GSK805 (2.5 μM). **(D)** Gene ontology analysis of 298 genes downregulated by all of the four compounds. Hypergeometric test and Benjamini-Hochberg p-value correction. **E.** GSEA plots depicting the enrichment of genes downregulated (1.5-fold) in cholesterol-biosynthesis pathway in MDA-MB-468 cell treated with XY018 (2.5 μM), GSK805 (2.5 μM), VTP-23 (2.5 μM and 10 μM) or TAK828F (2.5 μM and 10 μM) for 24 hours. FDR false-discovery rate. **F.** Heat map display of fold changes (in log2) in mRNA expression of 21 cholesterol-biosynthesis genes analyzed by RNA-seq in MDA-MB468 cells treated with different RORγ inhibitors for 24 hours. **G.** Heat map display of fold changes (in log2) in mRNA expression of 21 cholesterol-biosynthesis genes analyzed by qRT-PCR in HCC70 cells treated with 5 μM of different RORγ inhibitors for 48 hours. **H.** Heat map display of fold changes (in log2) in mRNA expression of KEGG 28 circadian rhythm genes analyzed by RNA-seq in MDA-MB468 cells treated with different RORγ inhibitors for 24 hours.



Supplementary figure 3.1 RORγ antagonists do not alter mRNA expressions of the major cholesterol biosynthesis genes in mouse Th17 cells

Naïve CD4+ T cells were isolated from C57BL/6 mice and activated under Th17-cell-polarizing conditions in the presence of different RORγ inhibitors at indicated concentrations (1 nM, 10 nM and 100 nM). After 4 days, *Hmgcr, Hmgcs1, Sqle and Mvk* mRNA expressions were measured by qRT-PCR.

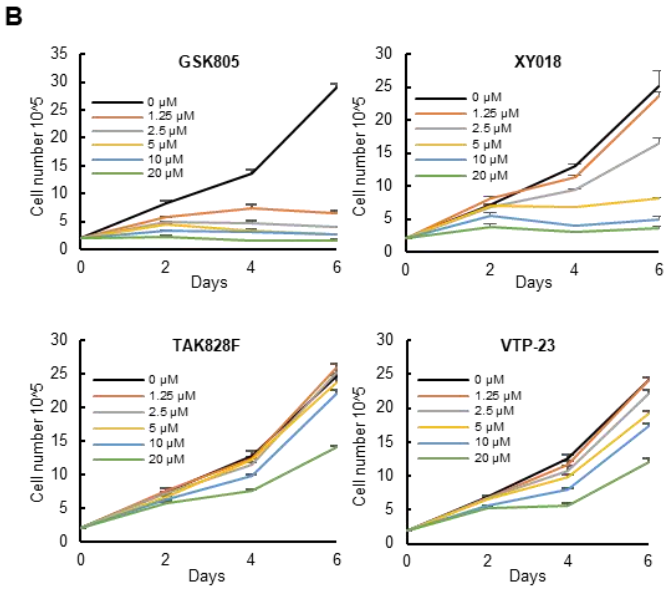
3.4.4 XY018 and GSK805, not VTP-23 or TAK828F, potently inhibit TNBC cell growth and survival

Notably, in contrast to their high potency in inhibiting cholesterol biosynthesis gene program in TNBC, XY018 and GSK805 have a relatively low activity in suppressing Th17-related cytokine production in Th17 cell, if compared with TAK828F and VTP-23. These data indicate a potential cell context-specific activity of different RORγ antagonists. To further examine their differential activities, we compared their effects on TNBC cell growth. Consistent with previous study [18], XY018 and GSK805 showed the lowest IC_{50} value in both HCC70 and MDA-MB-468 cells. TAK828F, VTP-23 and a RORγ agonist—LYC55716 all have an IC_{50} around or above 20 μM (Fig. 3.4A). Indeed, while 2.5 μM of GSK805 and XY018 displayed

strong inhibition on MDA-MB-468 cell growth, TAK828F and VTP-23 at concentrations below 20 μM did not significantly inhibit cell growth (Fig. 3.4B). The anti-growth activity of XY018 and GSK805 in MDA-MB-468 sustained for six days without further addition of the compounds. Similar inhibitory effects can be observed in a cell survival/colony-formation assay. While 5 μM of XY018 and 2.5 μM of GSK805 treatment was able to completely prevent colony formation of MDA-MB-468 cells, no effect can be observed when cells were treated with 5 μM of TAK828F or VTP-23 (Fig. 3.4C). These data indicate that GSK805 and XY018 but not TAK828F or VTP-23 potently inhibit TNBC cell growth. Together with the other results described above, we show that those structurally distinct RORγ antagonists possess cell context-specific activities in TNBC and Th17 cells.

A

IC_{50} (μM)	RORγ antagonist					RORγ agonist
	XY018	GSK805	SR2211	TAK828F	VTP-23	LYC55716
MDA-MB-468	4.03	2.37	10.11	>20	>20	>20
HCC70	2.22	7.68	7.55	>20	>20	>20



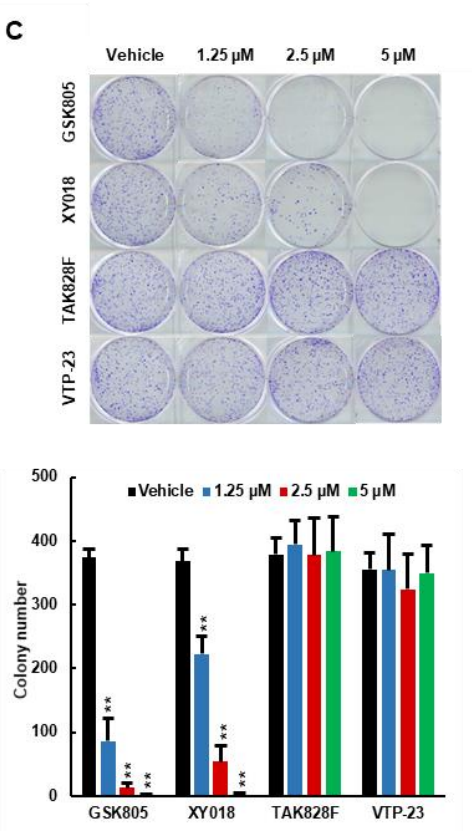


Figure 3.4 XY018, GSK805 and SR2211, not TAK828F or VTP-23, exhibit potent inhibition of TNBC cell growth and survival

A. The growth inhibition IC50 (μM) for RORγ antagonists XY018, GSK805, SR2211, TAK828F and VTP-23, or agonists LYC55716 in indicated TNBC cell lines treated for 4 days. **B.** MDA-MB468 cells were treated by

different RORγ antagonists as indicated. Two, four and six days later, viable cells were counted. **C.** MDA-MB468 cells were treated by different RORγ antagonists as indicated. Fourteen days later, representative images of colony formation were taken (top) and colonies were counted (bottom). Data are shown as mean ± SD. n = 3. Student's *t*-test. ** $p < 0.01$.

3.4.5 VTP-23 increases whereas XY018 reduces SREBP2 recruitment at chromatin targets of RORγ

We previously found that one of the major mechanisms of RORγ function in up-regulating tumor cholesterol biosynthesis is its novel activity of promoting recruitment of SREBP2, a well-known master regulator of cholesterol metabolism [7, 18]. Thus, to study the mechanism underlying the contrasting activities of the RORγ inhibitors in cholesterol biosynthesis gene expression, we performed ChIP analysis to measure their effects on the recruitment of SREBP2. Consistent with our published results [18], XY018 treatment and RORγ knockdown significantly reduced SREBP2 occupancy at the RORγ binding site of promoters of key cholesterol biosynthesis genes such as *HMGCS1, SQLE,* and *DHCR7* in the TNBC cells. In contrast, VTP-23 strongly increased occupancy of SREBP2 at the same RORγ target sites (Fig. 3.5A). Consistent with their contrasting effects on SREBP2 occupancy at the chromatin, we also observed that the transcriptional activation-linked histone mark H3K27ac was strongly increased by VTP-23 but reduced by XY018 at the promoter region of *HMGCS1*, *SQLE*, and *DHCR24* (Fig. 3.5B). Western blotting and qRT-PCR analysis indicated that the agonistic/activating effects on SREBP2 recruitment and H3K27ac mark by VTP-23 were not due to the compound effect on RORγ expression (Suppl Fig. 3.2).

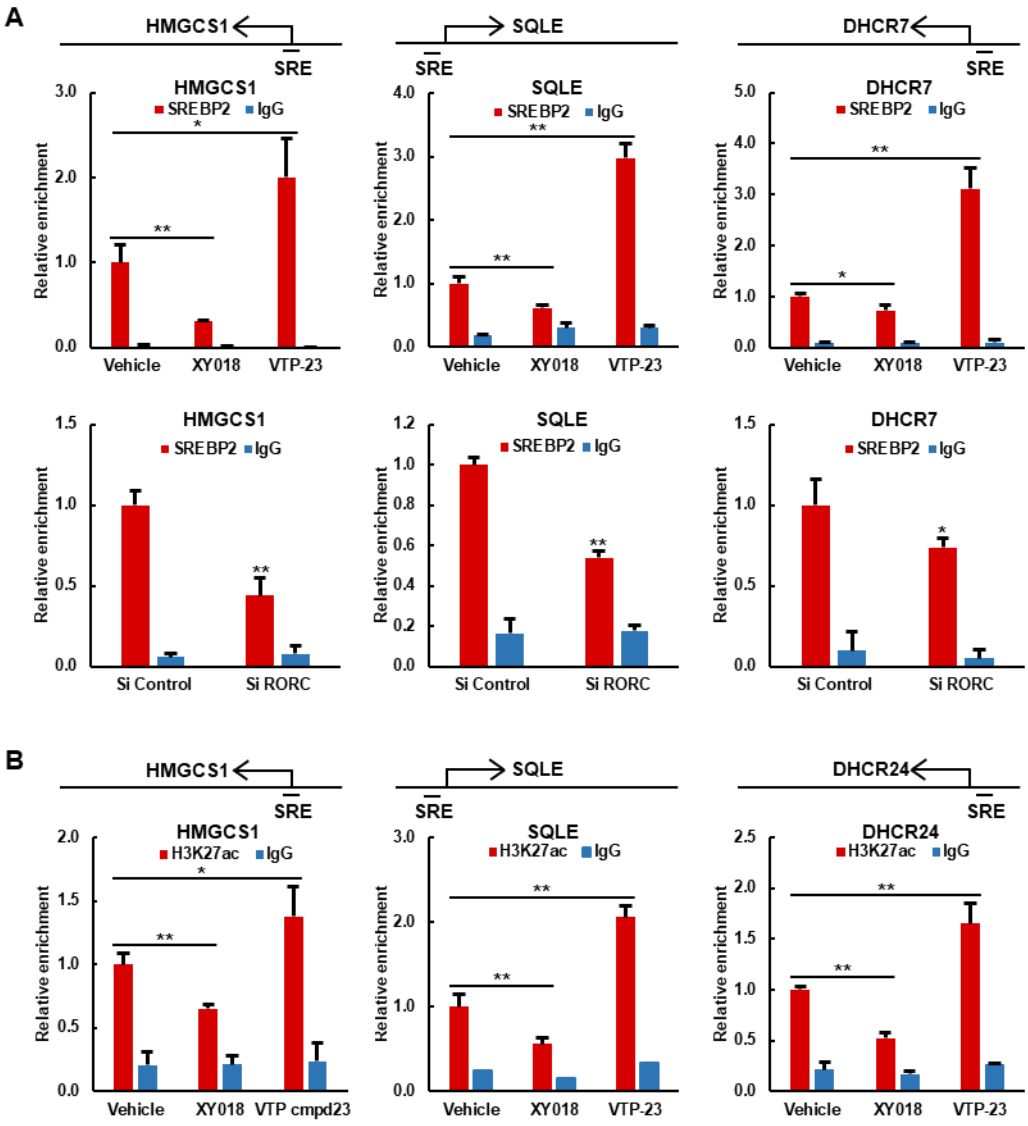
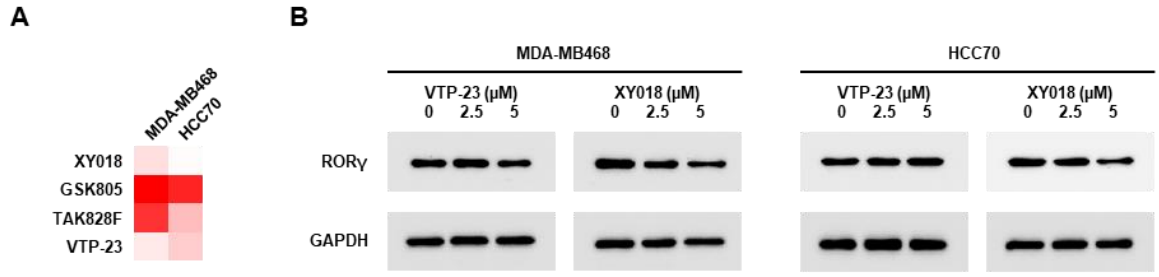


Figure 3.5 RORγ knockdown and antagonist treatment differentially alters SREBP2 recruitment and H3K27ac mark at RORγ binding sites of its target genes

A. ChIP-qPCR analysis of SREBP2 occupancy at the indicated RORγ binding sites of cholesterol biosynthesis genes in HCC70 cells treated by 5 μM of indicated RORγ antagonists (top) or transfected with siRORC (bottom) for 48 hrs. The RORγ binding sites and the genomic region analyzed by ChIP-qPCR are indicated by a short line close to the transcription start sites of each gene. **B.** ChIP-qPCR analysis of H3K27ac mark at the indicated sites of cholesterol biosynthesis genes in HCC70 cells treated by 5 μM of XY018 or VTP-23 for 48 hrs. n=3. Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.



Supplementary figure 3.2 RORγ antagonists affect RORγ expression in TNBC cells

A. Heat map display of fold changes (in log2) in mRNA expression of RORC gene analyzed by RNA-seq in MDA-MB468 cells treated with 2.5 μM of different RORγ inhibitors or by qRT-PCR in HCC70 cells treated with 5 μM of different RORγ inhibitors for 48 hours. **B**. Immunoblotting of RORγ in MDA-MB468 and HCC70 cells treated with VTP-23 or XY018 for 2 days. Representative blots. n=3.

3.4.6 Different RORγ antagonists display context-specific activity in altering chromatin accessibility at cholesterol biosynthesis gene loci

Many factors contribute to the control of transcriptional factor recruitment to DNA. Among them, one major determinant is chromatin accessibility which is regulated primarily by ATP-dependent chromatin remodeling complexes [31, 32]. Chromatin accessibility or openness in the cell can be measured by Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) [33]. We thus used ATAC-seq to measure the effects of the different RORγ antagonists on local chromatin accessibility. Consistent with the generally inhibitory effect of the antagonists on activator RORγ, both XY018 and VTP-23 significantly reduced genome-wide chromatin accessibility in the TNBC cells. Interestingly, XY018 caused a much greater reduction in the chromatin accessibility when compared with VTP-23 (Fig. 3.6A). More importantly, when we examined the effect on specific gene programs, we found that while XY018 strongly reduced the chromatin accessibility at the loci of cholesterol biosynthesis genes, little effect was observed in cells treated by VTP-23 (Fig. 3.6B). Consistent with our RNA-seq results, both XY018 and VTP-23 reduced chromatin accessibility at the loci of circadian rhythm genes. Similar to the effect on genome-wide chromatin accessibility, XY018

treatment resulted in a stronger reduction compared to VTP-23 (Fig. 3.6C). Our gene ontology (GO) analysis of genes that displayed ATAC-seq peak reduction by XY018 also revealed that cholesterol/steroid biosynthesis was among the significantly enriched programs (Fig. 3.6D). Indeed, treatment of XY018 markedly reduced chromatin accessibility at promoter and/or enhancer regions of major cholesterol biosynthesis genes, including *SQLE*, *HMGCS1*, *DHCR7* and *DHCR24* where RORγ occupancy can be detected by the RORγ ChIP-seq peaks [18]) (Fig. 3.6E, green arrows). In contrast, little alteration or even increased chromatin accessibility (indicated by red arrows) at the loci were observed when cells were treated with VTP-23. In addition, circadian rhythm was among the highly enriched programs when performing GO analysis of genes with reduced ATAC-seq peaks by either XY018 or VTP-23 treatment (Fig. 3.6D). Both XY018 and VTP-23 strongly decrease chromatin accessibility at promoter and/or enhancer regions of major circadian rhythm genes, such as *ARNTL* and *CRY1*. As expected, XY018 caused greater chromatin accessibility reduction compared with VTP-23 (Fig. 3.6F, green arrows). Together, these data strongly suggest that the RORγ antagonists possess context-specific activities in altering chromatin accessibility at RORγ binding sites in TNBC cells. While XY018 and VTP-23 both reduced chromatin accessibility at circadian rhythm gene loci, only XY018 potently decreased chromatin accessibility at cholesterol biosynthesis gene loci. Such distinctions in altering local chromatin structure are likely one of the underlying mechanisms for their distinct, context-specific activities in modulation of specific gene expression.

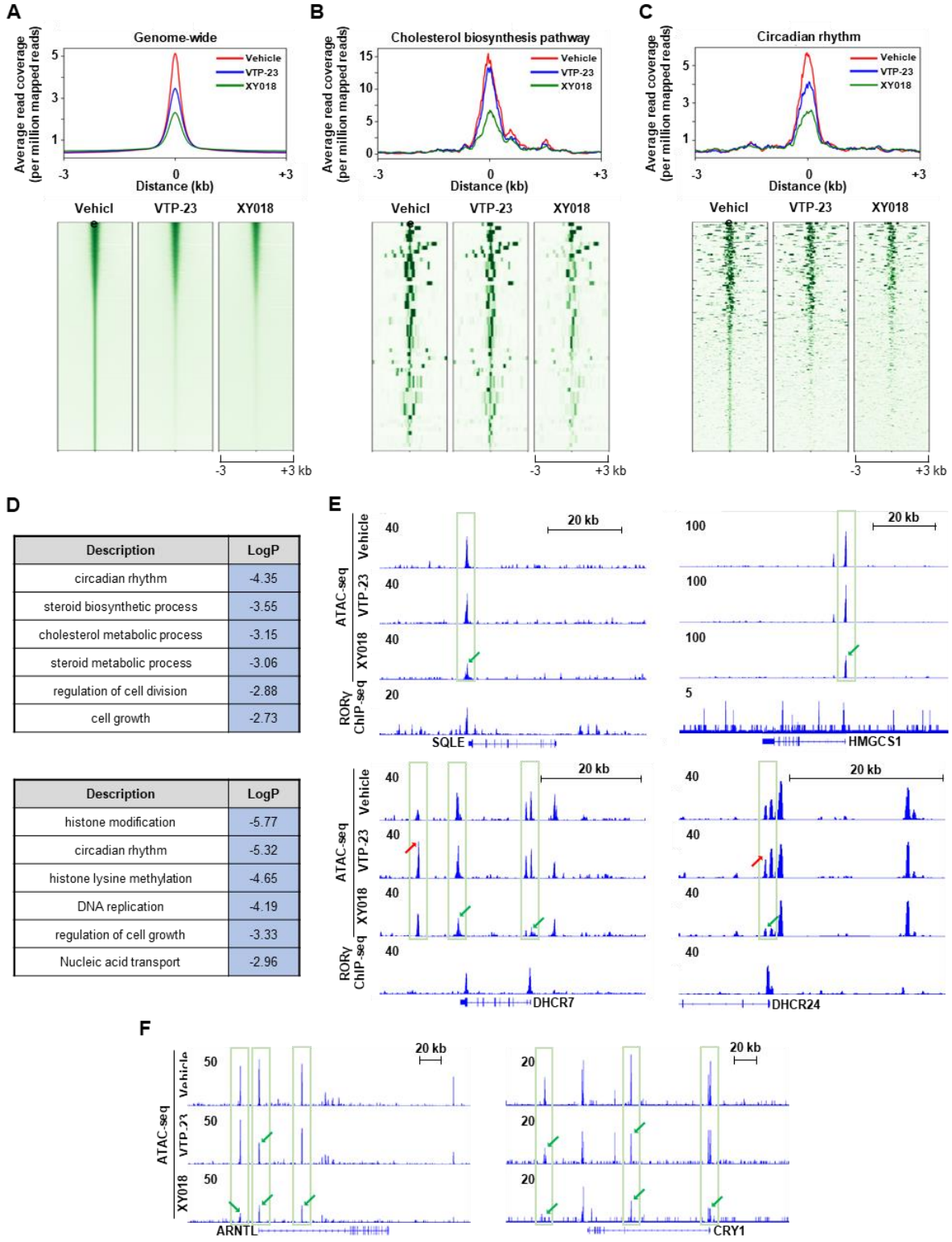


Figure 3.6. XY018 and VTP-23 differentially alters chromatin accessibility at cholesterol biosynthesis and circadian rhythm gene loci

A. Genome-wide ATAC-seq profile (top) and heat map (bottom) of genome-wide ATAC-seq signal intensity within ± 3 kb windows around the center of peak regions in MDA-MB-468 cells treated with vehicle, 5 μM of

VTP-23 or XY018 for 24 hours. **B.** ATAC-seq profiles (top) and heat map (bottom) of signal intensity within ± 3 kb windows around the center of peak regions of genes involved in cholesterol biosynthesis pathway in MDA-MB-468 cells treated as in (A). **C.** ATAC-seq profiles (top) and heat map (bottom) of signal intensity within ± 3 kb windows around the center of peak regions of genes involved in circadian rhythm in MDA-MB-468 cells treated as in (A). **D.** Gene ontology (GO) analysis of genes that displayed reduced ATAC-seq peaks compared with vehicle by XY018 (top) or VTP-23 (bottom) treatment in MDA-MB-468 cells. Hypergeometric and binomial test p value. **E.** IGV display of ATAC-seq and RORγ ChIP-seq signals in TNBC cells at representative cholesterol biosynthesis genes. Light green frames include the areas with the transpose access/open chromatin measurements significantly increased (red arrow) or decreased (green arrow) by the indicated treatments. **F.** IGV display of ATAC-seq signals in TNBC cells at representative circadian rhythm genes. Light green frames include the areas with the transpose access/open chromatin measurements significantly decreased (green arrow) by the indicated treatments.

3.4.7 Distinct chromatin accessibility alterations underlie the different activities of SR2211 and TAK828F in modulation of Il17 genes

To further examine context-specific activities of the RORγ inhibitors, we measured the activities of SR2211 and TAK828F in EL4 mouse lymphoma cells. EL4 cells express high levels of *Il17a* and *Il17f* upon stimulation by PMA and ionomycin and were used in the development of SR2211 [25, 34]. Consistent with published data [25], 5 μM SR2211 can significantly reduce *Il17a* and *Il17f* gene upregulation induced by PMA and ionomycin. Surprisingly, however, treatment of TAK828F further increased the mRNA level of *Il17a* and *Il17f* even after PMA and ionomycin stimulation (Fig. 3.7A). To understand how SR2211 and TAK828F act so differently in EL4 cells, we measured their effects on chromatin accessibility. Our ATAC-seq profiling of the entire chromatin regions of the RORγt-regulated Th17 signature genes revealed that after PMA and ionomycin stimulation, EL4 cells underwent a marked chromatin accessibility induction at loci of the signature genes [35, 36] (Fig. 3.7B). Importantly, we observed that TAK828F treatment further enhanced the induction of chromatin

accessibility (Fig. 3.7C) whereas SR2211 significantly reduced the induction at the signature genes as a whole (Fig. 3.7D). Indeed, the chromatin accessibility induction at promoter and/or enhancer regions of *Il17a*, *Il17f* and *Il23r* gene loci was reduced by SR2211 (Fig. 3.7E, green arrows). In contrast, TAK828F further enhanced the accessibility increase at those specific chromatin loci (Fig. 3.7E, red arrows). These results provided further evidence that different RORγ antagonists' distinct activities in specific cells are associated with their different effectiveness in altering chromatin accessibility. In addition, it also revealed a cell context-dependent opposing activity of TAK828F on a same RORγt target gene, namely TAK828F down-regulates IL-17A and IL-17F production in Th17 cells but up-regulates their expression in EL4 lymphoma cells.

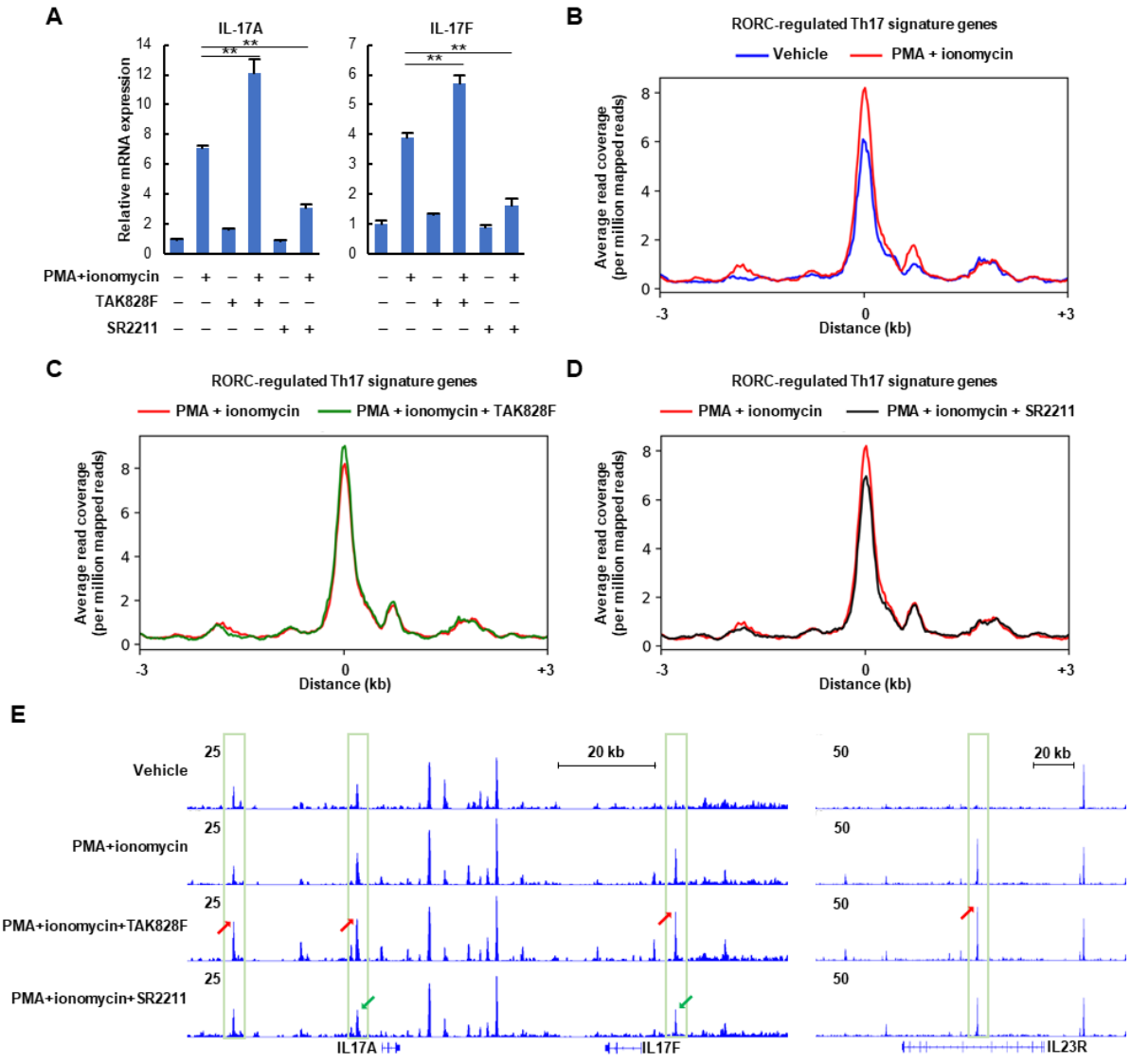


Figure 3.7 Opposing effects of SR2211 and TAK828F on IL-17 expression and chromatin accessibility/openness in EL4 lymphoma cells

A. qRT-PCR analysis of *Il17a* and *Il17f* gene expression in EL4 cells pretreated with 5 μM TAK828F or SR2211 for 20 hours, followed by stimulation with PMA and ionomycin for 4 hours. n=3. Student's *t*-test. ** $p < 0.01$. **B-D.** ATAC-seq profiles of signal intensity within ± 3 kb windows around the center of peak regions on RORγ-regulated Th17 signature genes (*Il17a*, *Il17f*, *Il21*, *Il22*, *Il23r*, *Ccr6*, *Ccl20*) in EL4 cells treated with vehicle, PMA and ionomycin stimulation, PMA and ionomycin stimulation together with 5 μM of TAK828F or SR2211. **E.** IGV display of ATAC-seq signals on *Il17a*, *Il17f* and *Il23r* gene loci in EL4 cells treated as in **(B)**. Light green frames include the areas with the transpose access/open chromatin measurements significantly increased (red arrow) or decreased (green arrow) by the indicated treatments.

3.5 Discussion

The initial identification of small molecules such as SR2211 and GSK805 that displayed strong antagonistic activities to RORγt in the autoimmune models provided a sustaining impetus to development of an ever-increasing number of RORγ inverse agonists/antagonists with diverse structures [7, 19]. In this study, we unexpectedly found that representative RORγ-targeting small molecules displayed remarkably distinct activities that are context-specific. For example, although VTP-23 and TAK828F can potently suppress the inflammatory gene expression in Th17 cells, they lack strong activities in suppressing tumorigenesis-linked programs such as cholesterol biosynthesis in TNBC cells that are controlled by RORγ. On the other hand, although GSK805 and XY018 are potent in inhibition of the cholesterol biosynthesis program, they are many folds less potent than the other two compounds in down-regulation of the inflammatory genes. Therefore, these compounds possess cellular context-specific activities in modulation of the RORγ or RORγt functions. Our other analyses also support this notion. Thus, consistent with the previous finding [25], SR2211 strongly inhibits the expression of *Il17a* and *Il17f* in EL4 mouse lymphoma cells. Intriguingly, in EL4 cells, TAK828F significantly up-regulates the expression of the two cytokines, suggesting an agonist activity to RORγt in the lymphoma cells. Likewise, on the expression of cholesterol biosynthesis genes, in contrast to the inhibitory effect displayed by GSK805, VTP-23 appears to stimulate their expression. The results that VTP-23 and TAK828F display apparently agonistic activities in specific cells and a highly potent antagonistic effect in the RORE-driven reporter assays in the TNBC cells strongly argue against the possibility that their lack of high potency in inhibition of cholesterol biosynthesis program is due to their inability to access the nucleus or the RORγ-associated regulatory complexes. In fact, VTP-23 is able to inhibit circadian rhythm gene program in the same cells where it induces cholesterol biosynthesis program, thus underscoring the gene context-specific modulation activities of the compound.

Overall, among the five compounds examined, the distinct activities in modulating RORγ functions displayed by a same small molecule such as TAK828F include a high potency of antagonist, a lack of significant effect and a significant stimulating effect or acting apparently as an agonist. Those distinct activities are displayed in the context of different cells (e.g., normal Th17 vs malignant T lymphoma or TNBC), different target programs (cholesterol biosynthesis vs circadian rhythm) or different target templates (e.g., plasmid-based reporters vs natural chromatin, or non-naturally iterated ROREs vs natural occurring sites). In our search for mechanisms that underlie their context-specific activities, we found that their activities in altering local chromatin structure at the target loci of RORγ or RORγt are closely linked to their distinct activities in regulation of the expression of RORγ target genes. Thus, XY018 strongly decreases the open chromatin structures at the cholesterol biosynthesis genes and the mRNA expression of those genes in the TNBC cells. In contrast, VTP-23 increases the open chromatin structure at specific gene loci and stimulates their expression. Likewise, SR2211 decreases PMA-ionomycin-induced open chromatin structures and the expression of *Il17a* and *Il17f* genes whereas TAK828F increases the chromatin structure and the expression of the *Il17* genes. Chromatin accessibility is a key determinant of transcription factor recruitment to their target sites. Indeed, we found that RORγ compounds such as VTP-23 that induce RORγ-controlled cholesterol biosynthesis genes in TNBC cells also increase the recruitment of master regulator SREBP2 whereas compounds such as XY018 that inhibit the cholesterol gene program reduces SREBP2 recruitment at chromatin targets of RORγ.

Currently, it is unclear how different RORγ modulators targeting the same receptor in the same cell can elicit such highly distinct effects on the local chromatin structure and recruitment of SREBP2. Chromatin accessibility can be regulated by various co-factors, including ones that possess chromatin modifying (e.g. histone acetylation or methylation) or de-modifying enzymatic activities, and ATP-dependent chromatin remodeling activities [37]. RORγ can

interact several co-activators, including SRC/NCOA1-3 and p300, and also with co-repressors, including NCOR1, NCOR2, RIP140 [38]. A recent study demonstrates that a component of SWI/SNF complex is crucial for the induction of *IL17* genes in Th17 cells and also for RORγt-dependent histone activation marks, suggesting a functional interplay between the chromatin remodeling complex and the histone modification complexes in RORγ function [39]. One possibility for the different RORγ modulators to exert distinct effects at the local chromatin is their ability to induce different conformational changes to the receptor which in turn leads to the recruitment (or in some cases, lack of recruitment) by the liganded receptor of specific protein complexes with the chromatin modifying or remodeling activities. The other possibility is that the distinct conformational change leads to association or dissociation of proteins or complexes such as other transcription factors or co-factors that are already assembled at the RORγ target sites. Even for the same chromatin locus (e.g., *Il17a/f*), different cells can have distinctive chromatin structure and associated protein complexes. Therefore, the impact of the distinct conformational change in RORγ protein on the association and function of the local protein complexes will likely be different.

NR superfamily represents a unique paradigm for identification of tissue-selective modulators. Many selective modulators have been identified for ER-α, AR and GR [5, 40]. Indeed, raloxifene, a second generation SERM, is effective in reducing the risk of breast cancer by antagonizing the ER function in breast tissue, whereas it prevents bone loss by acting as an ER agonist and lacks agonist activity in the uterus [41, 42]. Several selective GR modulators (SEGRMs) have also been identified [43]. Drugs that selectively enhance the trans-repression function of GR without significant effect on its transactivation have been in clinical trial for inflammatory diseases. Several selective AR modulators (SARMs) displayed potential therapeutic values for treatment of cachexia, osteoporosis, prostate cancer and AR+ breast cancer [44-48]. However, currently no selective modulators have been reported for RORs.

RORγt plays a critical role in the differentiation and function of subsets of immune cells such as Th17 [49]. On the other hand, recent findings that RORγ in tumor cells plays important roles in promoting tumor growth and metastasis would pose significant concerns for potential use of agonist of RORγ in treating cancer. Therefore, an ideal RORγ modulator for treatment of autoimmune diseases will selectively suppress the heightened RORγt functions in the subset of T cells. Likewise, antagonists targeting RORγ for cancer therapy will require the modulators being selective to the tumor cells and sparing the normal function of RORγ(t) in the host immune system. Here we report evidence that several RORγ modulators identified initially as inverse agonists or antagonists display cell-selective modulator activities. Further studies involving additional animal work are needed to demonstrate whether they or any other inverse agonists/antagonists of RORγ indeed possess properties of tissue-selective modulator. Nonetheless, our results strongly suggest that development of tissue-selective RORγ modulators or SERORMs for effective and safe treatment of diseases such as autoimmune and cancer is not only desirable but also readily attainable.

3.6 Conclusion

In summary, our study shows that inverse agonists/antagonists of RORγ possess different or even contrasting activities in regulating RORγ targeted gene programs in tissue/cell-specific manner. Our findings also highlight that the activities at natural chromatin are key determinants of RORγ modulators' tissue selectivity.

3.7 Acknowledgement

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3.8 Supplementary Materials

Table 3.1 Primers used for qPCR and ChIP assay

Primers for mouse qRT-PCR	
Gapdh-F	GGTGGACCTCATGGCCTACA
Gapdh-R	CTCTCTTGCTCAGTGTCCTTGCT
Il17a-F	CTCCAGAAGGCCCTCAGACTAC
Il17a -R	AGCTTTCCCTCCGCATTGACACAG
Il17f -F	GCCCAGTCTCTTTGTGTTAG
Il17f -R	CCATGTGTGCTTCTTCCTT
Hmgcr-F	ATGGCTGGGAGCATAGGCGG
Hmgcr-R	CTGCATCCTGGCCACATGCG
Hmgcs1-F	AGGAACGTGGTATCTGGTCA
Hmgcs1-R	TGTGTTACTATGCACGAGCC
Sqle-F	CCGTTTACAGCCAGGCGAGC
Sqle-R	ACTGATGGACACGGGCCTCT
Mvk-F	AGGTCCCGCGGAGTACCAAG
Mvk-R	CTAGCACGCGCTCACACTCC
Primers for human qRT-PCR	
GAPDH-F	GCAGGGATGATGTTCTG
GAPDH-R	GTATGACAACAGCCTCAA
ACAT2-F	GTGCTGCAGCTGTCGTTCTTAT
ACAT2-R	CTTCCAGTGACCAACCTGCTTT
HMGCS1-F	CTTTCGTGGCTCACTCCCTTTC
HMGCS1-R	AGGGCAACAATTCCCACATCTT
HMGCR-F	GCACCAAGAAGACAGCCTGAATAG
HMGCR-R	TCTGAGGAGTCTGCATGGAAAGA
MVK-F	CCTCAGCTTACCCAACATTGGTATC
MVK-R	TCCGGCAGATGGACAGGTATAA
PMVK-F	AGTGGTTTCGGGAGGCCTAT
PMVK-R	TCAGGTTCTCCAACTGCTCCT
MVD-F	TGGCATCGGTGAACAACTTCC
MVD-R	CCCATCTGCCACTCCACAAAG
IDI1-F	CACACCCTGGATATGTGTTCTGTTT
IDI1-R	TCTGCAAGTGCTCCGGAAATG
GGPS1-F	GGCAGTTCCAAGCCAGTTTCTA
GGPS1-R	CCTCCCAAAGTGCTGGGATTAC
FDPS-F	GCCAAGGAAACAGGATGCTGATAG
FDPS-R	AGCTTCAGCAGGCGGTAGATA
FDFT1-F	GGTCCCGCTGTTACACAACTTT
FDFT1-R	GCCATCCCAATGCCCATTCT
SQLE-F	CCATGCTCCACTGACTGTTGTT
SQLE-R	AGATGAGAACTGGACTCGGGTTAG
LSS-F	GCGAGGAGCGGCGTTATTT
LSS-R	TGTAGGAGATGGCACAGGACTT
CYP51A1-F	TTGGCTGCCTTTGCCTAGTT
CYP51A1-R	GCTGCCCTGCCAAGAGTAAT

TM7SF2-F	CACCCTCACCGCTTTCATCTT
TM7SF2-R	CGGGTCGCAGTTCACAGAAATA
SC4MOL-F	CCTCCCAAAGTGCTGGGATTAC
SC4MOL-R	AAGTTCTTGGTGCCGGCTTT
NSDHL-F	GCCAGGAACGGCAAGATGAA
NSDHL-R	GCGAGACAGGAATGTCCAGAAAG
HSD17B7-F	CCACCACTGGCTTTGGAAGAA
HSD17B7-R	GCCTCCCAAAGTGCTGGAATTA
EBP-F	ACTGGCCTCAGCACCTAAGA
EBP-R	AACCCACACACTGCAAACCA
SC5D-F	GTTGCACCATCCCTGGTTTCT
SC5D-R	CTGCCCTCTGCAACTGATTTCT
DHCR7-F	CATTGACATCTGCCATGACC
DHCR7-R	ACAGGTCCTTCTGGTGGTTG
DHCR24-F	TGTTGCCTGAGCTTGATGAC
DHCR24-R	GACCAGGGTACGGCATAGAA
Primers for ChIP-qPCR	
HMGCS1-F	GCCGTATCTCGCAGCTCCGTCA
HMGCS1-R	GCCGGTAGAGTTGCCCGGCAG
SQLE-F	GCTAGCGGAATGAATGGAAACG
SQLE-R	ACTGAGGTGTGCTCGCTTTG
DHCR7-F	CCCGGATGATGTCAGCGATT
DHCR7-R	GCCTGGGCCGTCAATCT
DHCR24-F	CTGCTGAGCTTGAACACCAC
DHCR24-R	GAGTTCGTGCTCATCCACCA

Table 3.2 Antibodies used in immunoblotting

Antibody	Vendor	Catalog number	Dilution
RORγ	Ebioscience	14-6988-82	1:1000
GAPDH	Cell signaling	#2118	1:2000

3.9 Reference

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Chapter 4

Natural compounds ursolic acid and digoxin exhibit inhibitory activities to cancer cells in RORγ-dependent and -independent manner

4.1 Abstract

Natural compounds ursolic acid (UA) and digoxin isolated from fruits and other plants display potent anti-cancer effects in preclinical studies. UA and digoxin have been at clinical trials for treatment of different cancers including prostate cancer, pancreatic cancer and breast cancer. However, they displayed limited benefit to patients. Currently, a poor understanding of their direct targets and mechanisms of action (MOA) severely hinders their further development. We previously identified nuclear receptor RORγ as a novel therapeutic target for castration-resistant prostate cancer (CRPC) and triple-negative breast cancer (TNBC) and demonstrated that tumor cell RORγ directly activates gene programs such as androgen receptor (AR) signaling and cholesterol metabolism. Previous studies also demonstrated that UA and digoxin are potential RORγt antagonists in modulating the functions of immune cells such as Th17 cells. Here we showed that UA displays a strong activity in inhibition of RORγ-dependent transactivation function in cancer cells, while digoxin exhibits no effect at clinically relevant concentrations. In prostate cancer cells, UA down-regulates RORγ-stimulated AR expression and AR signaling, whereas digoxin up-regulates AR signaling pathway. In TNBC cells, UA but not digoxin alters RORγ-controlled gene programs of cell proliferation, apoptosis and cholesterol-biosynthesis. Together, our study reveals for the first-time that UA, but not digoxin, acts as a natural antagonist of RORγ in the cancer cells. Our finding that RORγ is a direct target of UA in cancer cells will help select patients with tumors that likely respond to UA treatment.

4.2 Introduction

Natural products isolated from plants or microorganisms are excellent sources for novel drug discovery [1,2]. These naturally active products have relatively high oral bioavailability,

special biological activities and known insights of safety and efficacy. Among the natural products, ursolic acid (UA) and digoxin have been reported to possess anti-cancer activity by disrupting multiple signaling pathways. UA is a pentacyclic triterpenoid presented in plants, fruits and herbs, including apple, basil and rosemary. It can inhibit NF-κB and STAT3 signaling [3,4], and activate JNK-mediated apoptosis [5] in prostate cancer cells and tumors. UA also down-regulates STAT3 [6] and FoxM1 [7] signaling pathways in breast cancer cells. Digoxin can be isolated from foxglove plant. It potently inhibits the sodium potassium adenosine triphosphatase (Na+/K+ ATPase) and is clinically used for heart diseases, including atrial fibrillation and heart failure. Recent studies suggest that digoxin can display anti-proliferation activity in cells of prostate cancers and breast cancers [8,9]. Both UA and digoxin have been evaluated in ongoing or completed clinical trials for treatment of several types of cancer. However, their direct targets in cancer cells and tumors remained unclear.

UA has been reported as an inhibitor of amyloid β interaction with its receptor CD36 [10]. Largely based on results from reporter gene assays, UA was also characterized as modulators of members of the nuclear receptor (NR) family of transcription factors, specifically as agonist of PPARα [11], and antagonist of LXRα [12] and RORγt, a T cell-specific isoform of RORγ [13]. UA strongly reduces IL-17 expression in naïve CD4+ T cells and blocks the differentiation of T helper 17 (Th17) cells. Interestingly, UA does not appear to modulate the function of RORα, which is another member of the ROR subfamily of NR. In an early search for modulators of RORγt, digoxin was also shown to possess antagonistic activities to RORγt in Th17 cells [14]. Thus, UA and digoxin are the two major natural compounds that were identified as modulators of RORγt in the early studies [13,14].

Recently, in search for alternative therapeutic targets for advanced cancer, RORγ in tumor cells was identified to play a critical role in tumor progression in certain types of cancer [15], including castration-resistant prostate cancer (CRPC) [16,17,18,19], triple-negative breast

cancer (TNBC) [20,21], small cell lung carcinoma (SCLC) [22] and pancreatic ductal adenocarcinoma (PDAC) [23]. In CRPC tumors and cells, RORγ directly activates androgen receptor (AR) expression and AR signaling [16,17,19]. In TNBC cells and tumors, RORγ acts as a master activator of tumor cholesterol biosynthesis program [20,21]. Pharmacological and genetic inhibition of RORγ strongly block prostate cancer (PCa) and TNBC cell growth and metastasis, suggesting that RORγ is a novel therapeutic target for cancer [16,17,18,19,20,21]. Although a large number of synthetic, small-molecule modulators of RORγ/RORγt have been identified [15,24], few studies made a direct comparison of their activities. Recently, it was reported that structurally distinct, small-molecule modulators can display large differences in their activities in altering the function of RORγ/RORγt in control of its target gene expression [21]. Here we examined the activities of UA and digoxin in cells of PCa and TNBC where the function of RORγ is relatively defined [16,20]. We found that UA but not digoxin disrupted the previously defined, RORγ-targeted gene programs. Our results demonstrate that UA, not digoxin, acts as a natural antagonist of RORγ in PCa and TNBC cells.

4.3 Materials and Methods

Cell culture

C4-2B, 22RV1, LNCaP, PC3 and HCC70 cells were cultured in RPMI1640 (Corning) supplemented with 10% FBS. DU145 and MDA-MB-468 cells were cultured in DMEM (Corning) supplemented with 10% FBS. Cells were grown at 37 °C in 5% CO2 incubators. Cells were obtained from ATCC and were regularly tested being negative for mycoplasma.

Chemicals

XY018 (purity > 99%) was synthesized by WuXi AppTec. Ursolic acid (purity > 95%) and digoxin (purity > 98%) were purchased from Cayman.

Cell viability, proliferation and colony formation

For cell viability, cells were seeded in 96-well plates at 1000-2000 cells per well in a total volume of 100 µL of media. After 4 days of incubation of compounds, Cell-Titer Glo reagents (Promega) were added, and luminescence was measured on Varioskan™ LUX multimode microplate reader (Thermo Scientific), according to the manufacturer's instructions. All experimental points were set up in triplicate, and the entire experiments were repeated three times. The estimated in vitro IC50 values were calculated by using GraphPad Prism 9 software. For cell proliferation, cells were seeded in 6-well plates at 2 x 10^5 per well and treated as indicated. Total viable cell numbers were counted using Countess™ II Automated Cell Counter (Invitrogen). For colony formation assay, 500 cells were seeded in a well of 6-well plate and cultured for 21 days with the medium changing every 5 days. When the cell clone grew visible, the medium was removed, and the cells were fixed with 10% formalin for 10 mins. The plated were washed with PBS for two times, and cell colonies were stained with 0.2% crystal violet (in 10% formalin) for 30 mins. The above assays were performed in duplicates, and the entire experiments were repeated three times.

Luciferase reporter gene assay and plasmid transfection

Transient transfection and reporter-gene assays were performed as previously described with modification [21]. Briefly, cells were co-transfected with pLX304-RORγ or empty vector and 7 X RORE reporter plasmid using lipofectamine 3000 (Cat. L3000015, Invitrogen). Renila plasmid was co-transfected for normalization. After 12 hours of incubation, cells were treated with vehicle or different compounds as indicated for another 24 hours. The luciferase activity was analyzed using Dual-Glo Luciferase Assay System (Promega) on Varioskan™ LUX multimode microplate reader (Thermo Scientific), according to the manufacturer's instructions. All transfections were performed at least in triplicate, and each experiment was repeated three times.

qRT- PCR and western blotting analysis

Total RNA was isolated from cells using TRIzol™ Reagent (Cat. 15596018, Invitrogen). The cDNA was prepared using qScript™ cDNA SuperMix (Cat. 95048-100, QuantaBio). Quantitative PCR were performed as previously described with modification [25]. Briefly, cDNAs were mixed with SYBR Green qPCR master mix (Cat. A25742, Applied Biosystems) and gene specific primers. The PCR were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). The fluorescent values were collected, and fold difference was calculated. GAPDH was used as the internal reference to normalize the relative level of each transcript. The experiments were performed at least three times. Primers are listed in Supplementary Table 4.1.

After cells were lysed, protein concentrations were measured and adjusted using DC™ Protein Assay Kit II (Cat. 5000112, Bio-Rad). Proteins were separated by SDS-PAGE gel and transferred onto PVDF membranes (Cat. IPVH00010, MilliporeSigma). Membranes were incubated with indicated primary antibodies at 4 ℃ overnight and then subjected to second antibody incubation. Antibody-recognized proteins were visualized using ChemiDocTM MP imaging system (Bio-Rad) after incubation with HRP substrate (Cat. WBLUR0500, MilliporeSigma). Antibodies used are shown in Supplementary Table 4.2.

RNA-seq and bioinformatics analysis

HCC70 cells were treated as indicated before RNA extraction. RNA-seq libraries from 1 μg total RNA were prepared and validated as previously described [25]. Sequencing was performed on an Illumina HiSeq 2000 Sequencer at BGI Tech (Hong Kong). The FASTQ-formatted sequence data were analyzed using a standard BWA-Bowtie-Cufflinks workflow. Briefly, sequence reads were aligned to the reference human genome assembly (hg38) with BWA and Bowtie software. Subsequently, the Cufflinks package [26]. was applied for transcript assembly and quantification gene expression. To avoid spurious fold levels due to

low expression values, only subsets of genes that have expression value of RPKM (reads per kilobase per million mapped reads) or FPKM (fragments per kilobase of exon model per million mapped reads) above 1 for either the vehicle treated cell, or the compound treated cells are included. GSEA was performed using the Java desktop software (http://www.broadinstitute.org/gsea) as described previously [27]. Genes were ranked according to the shrunken limma log2 fold changes and the GSEA tool was used in 'pre-ranked' mode with all default parameters. Previous reported AR-activity signature genes [28] were used in the GSEA analysis.

ChIP-seq data analysis

ChIP-seq assay was performed as previously described. [20].

Fastq files from ChIP-seq were processed by the pipeline of AQUAS Transcription Factor and Histone (https://github.com/kundajelab/chipseq_pipeline). Briefly, sequencing tags were mapped against the Homo sapiens (human) reference genome (hg19) by using BWA 0.7.1551. Uniquely mapped tags after filtering and deduping were used for peak calling by model-based analysis for ChIP-Seq (MACS; 2.1.0) to identify regions of enrichment over background. Normalized genome-wide signal-coverage tracks from raw-read alignment files were built by MACS2, UCSC tools (bedGraphToBigWig/bedClip; http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/), and bedTools (https://github.com/arq5x/bedtools2). Visualization of the ChIP-seq signal at enriched genomic regions (avgprofile and heatmap) was achieved by using deepTools (https://deeptools.readthedocs.io/en/develop/index.html).

Statistical analysis

Cell culture-based experiments were performed three times or more, with assay points triplicated. The data are presented as mean values ± SD. Statistical analyses were performed by GraphPad Prism software 9.

4.4 Results

4.4.1 Ursolic acid (UA) and digoxin differs in effectiveness of killing cancer cells

To compare the anti-growth and -survival activities in cancer cells of UA and digoxin with synthetic RORγ inhibitors, we included XY018, which was characterized in its activity in antagonizing the function of RORγ in control of gene programs in the cancer cells and tumors [16,20,22]. In the PCa and TNBC cells, UA displayed slightly weaker but comparable inhibitory activity in modulating cell growth and survival when compared to XY018. Specifically, XY018 displayed an IC50 of 2 to 6 μM in the two PCa cell lines (C4-2B and 22RV1) and the two TNBC cell lines (HCC70 and MDA-MB-468), while UA showed an IC50 of 7 to 10 μM for the same cell models. On the other hand, Digoxin displayed an IC50 in the sub-micromolar range for both PCa and TNBC cells with IC50 values over 50 fold lower than those of XY018 in the PCa cells (Fig. 4.1A). Similar differences in their effectiveness were observed in assays of cell numeration and cell survival/colony formation assay (Fig. 4.1B and C).

4.4.2 UA but not digoxin blocks transactivation activity of RORγ in cancer cells

To examine whether the anti-growth effects of UA and digoxin is associated with their inhibition of RORγ functions in cancer cells, we performed luciferase reporter assay in different cancer cells. In PCa (C4-2B and 22Rv1) and TNBC cells (HCC70), UA diminished the RORγ-dependent activation of the reporter in a concentration-dependent manner (Fig. 4.2A). Specifically, more than 70% inhibition of its transactivation was observed when cells were treated with 1 μM UA. However, no significant inhibitory effect was observed when cells were treated with digoxin (Fig. 4.2A) at concentrations that display potent cell growth inhibition as shown in Fig. 4.1. Additionally, the protein expression of RORγ remained unchanged when cells were treated with UA or digoxin (Fig. 4.2B). Together, these data

suggest that UA and digoxin may inhibit cancer cell growth through RORγ-dependent and -independent mechanisms.

A

IC50(μM) (mean±SD)	Prostate Cancer cells		TNBC cells	
	C4-2B	22RV1	HCC70	MDA-MB-468
XY018	4.06±0.77	6.53±1.07	2.22±0.86	4.03±1.61
Ursolic acid	8.51±0.34	7.76±0.47	9.73±0.36	10.46±0.07
Digoxin	0.065±0.016	0.094±0.014	0.642±0.218	0.273±0.016

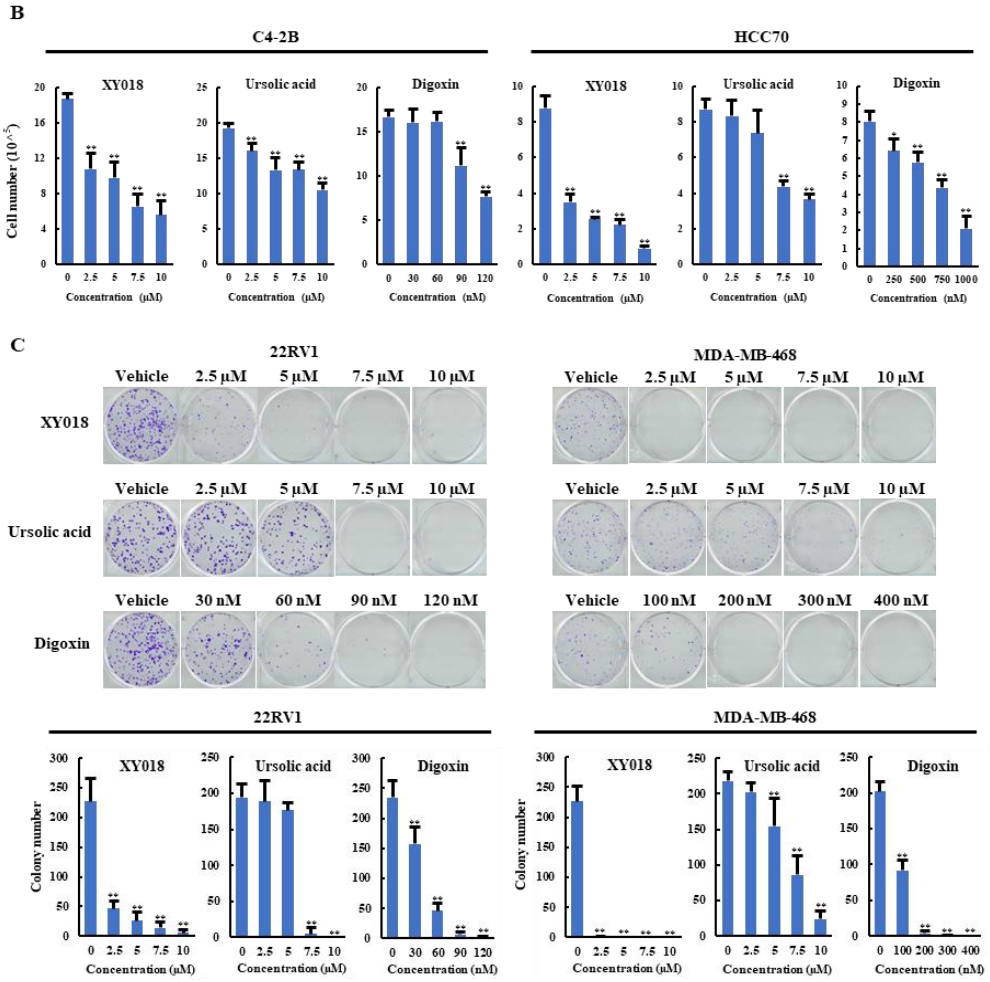

Figure 4.1 UA and digoxin display different effectiveness in inhibition of cancer cell growth when compared to synthetic RORγ antagonist XY018

A. The growth inhibition IC50 (μM) for synthetic RORγ antagonist XY018, natural RORγ antagonists ursolic acid and digoxin in indicated PCa and TNBC cell lines treated for 4 days. **B**. C4-2B and HCC70 cells were treated by different RORγ antagonists as indicated. Viable cells were counted after four days. Data are shown as mean ± SD. n = 3. Student's t-test. * $p < 0.05$, ** $p < 0.01$. **C**. 22RV1 and MDA-MB-468 cells were treated by different RORγ antagonists as indicated. Fourteen days later, representative images of colony formation were taken (top) and colonies were counted (bottom). Data are shown as mean ± SD. n ≥ 3. Student′ s t-test. ** $p < 0.01$.

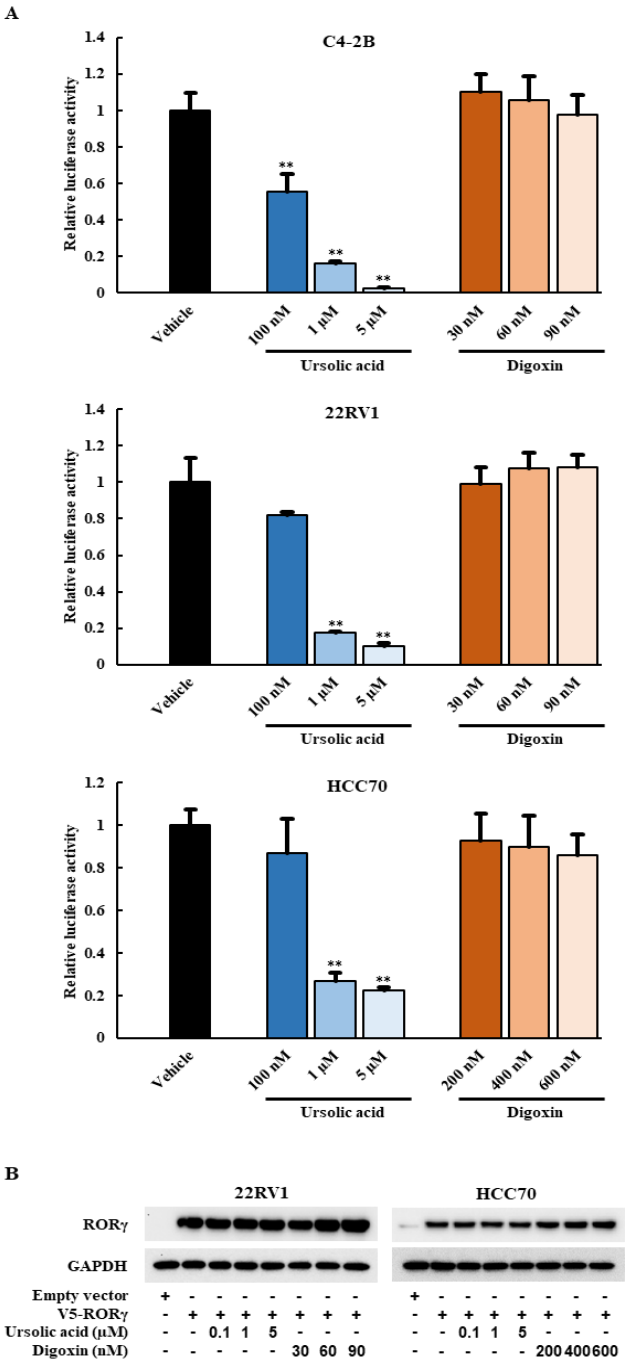

Figure 4.2 UA but not digoxin blocks transactivation function of RORγ in PCa and TNBC cells

A. 7X-RORE luciferase reporter activity changes by treatment of ursolic acid or digoxin in C4-2B (top), 22RV1 (middle) or HCC70 (bottom) cells for 24 hours. Normalized luciferase activity from cells treated with vehicle and transfected with RORγ-expressing plasmid were set as 1. Data are shown as mean ± SD. $n \geq 3$. Student's t-test. ** $p < 0.01$. **B**. Immunoblotting of 22RV1 and HCC70 cells transfected with V5-RORC expression vector. Twelve hours after transfection, cells were treated with UA or digoxin at indicated concentration for another 24 h.

4.4.3 UA but not digoxin disrupts RORγ-mediated AR expression and AR signaling

In our previous studies, we demonstrated that RORγ directly activates AR gene expression and that synthetic RORγ antagonists reduce the expression of AR and its variant AR-V7 and AR-controlled gene programs in PCa cell lines and tumors [16,17]. To further examine whether the anti-growth effect of UA in PCa is through RORγ, we performed RNA-seq analysis of C4-2B cells treated by 10 μM UA. Gene-set enrichment analysis (GSEA) showed that an AR target gene signature gene set [28] was significantly disrupted by UA treatment at 24 and 48 hours (Fig. 4.3A, top panels). In contrast, 48 hours of digoxin treatment significantly enhanced the expression of the AR target gene signature (Fig. 4.3A bottom right panel. Indeed, although some of the previously classified androgen-induced genes such as KLK2 and KLK3 [28] were inhibited by both UA and digoxin treatment, AR and other AR-regulated genes like FKBP5 were downregulated by UA while upregulated by digoxin treatment (Fig. 4.3B). Consistent with RNA-seq analysis, UA reduced protein expressions of AR and its variant AR-V7 in C4-2B and 22RV1 cells. Conversely, digoxin treatment had no effects or slightly increased AR expression in C4-2B or 22RV1 cells, respectively (Fig. 4.3C).

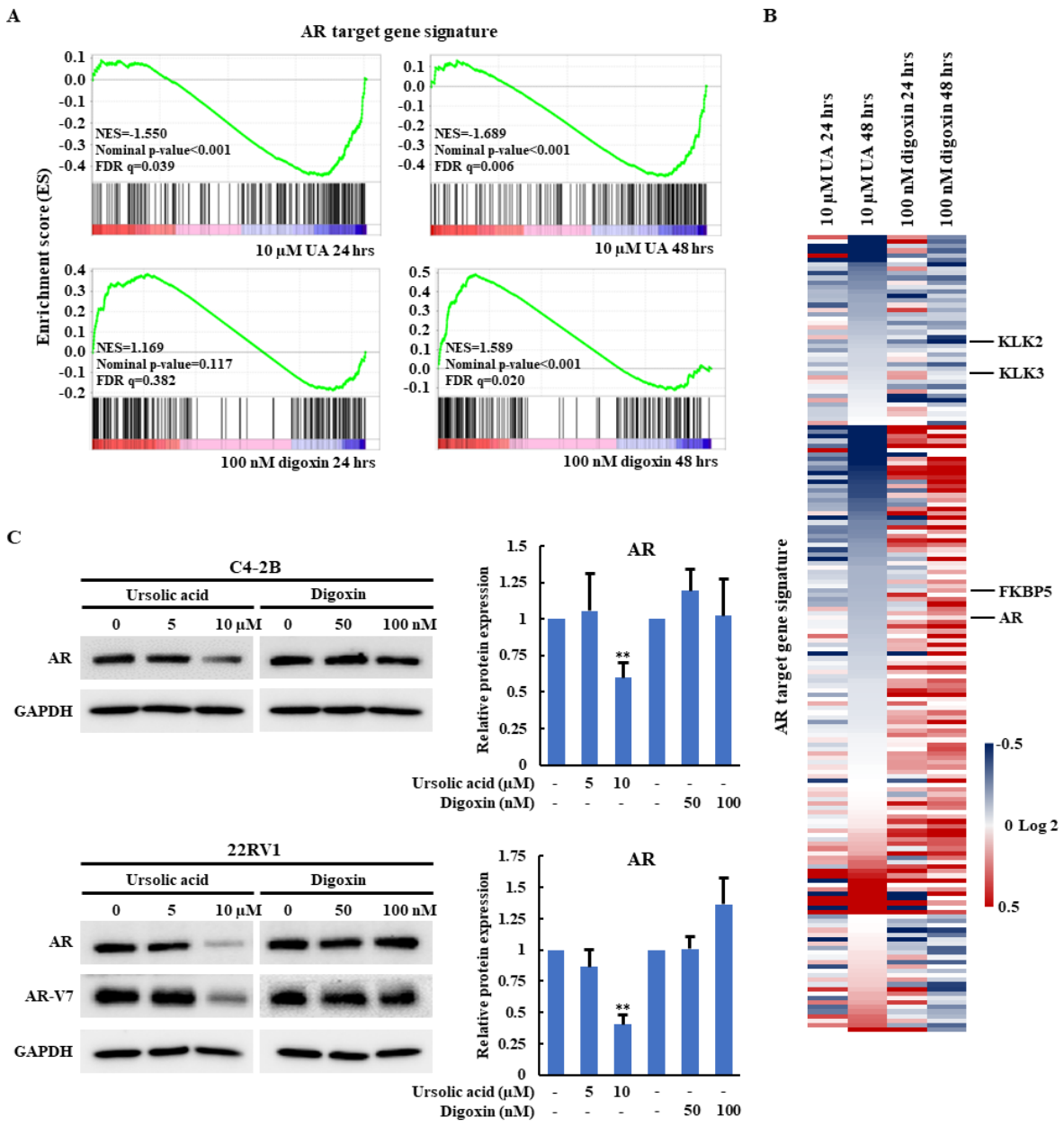

Figure 4.3 UA but not digoxin inhibits AR expression and AR-signaling in PCa cells

A. GSEA of the AR signaling pathway in C4-2B cell treated with UA (10 μM) or digoxin (100 nM) for 24 or 48 hours. NES, normalized enrichment score. FDR, false-discovery rate. **B.** Heat map display of fold changes (in log2) of AR-signature gene mRNA analyzed by RNA-seq in C4-2B cell treated with UA (10 μM) or digoxin (100 nM) for 24 or 48 hours. **C.** Immunoblotting of AR (full length) in C4-2B cell, AR (full length) and AR-V7 in 22RV1 cell treated with UA or digoxin at indicated concentration for 48 hours (left). Quantification of western blotting (right). AR expressions were normalized to that of GAPDH. Data are shown as mean ± SD. n ≥ 3. Student's *t*-test. ** $p < 0.01$.

4.4.4 UA displays potent anti-proliferation activity in AR-positive but not AR-negative PCa cells

To further examine whether UA inhibits PCa cell growth through disrupting AR signaling, we compared the anti-cancer effects of UA and digoxin in AR-positive and -negative PCa cells. As expected, UA showed significant stronger inhibitory activity in AR-positive PCa cells compared to AR-negative PCa. Specifically, 5 and 7.5 μM of UA was sufficient to strongly inhibit AR-positive LNCaP and 22RV1 cell growth, while 10 μM of UA had little or no effect on AR-negative DU145 and PC3 cell proliferation (Fig. 4.4A). In contrast, digoxin displayed similar anti-growth effects in both AR-positive and AR-negative PCa cell lines (Fig. 4.4B). In line with the cell growth effects, UA treatment potently reduced the protein expressions of key cell proliferation genes, including C-MYC, Cyclin A, Cyclin D1 and Cyclin E in LNCaP, but not in AR-negative PCa cell lines (PC3 and DU145). Additionally, expressions of critical cell apoptosis genes including cleaved Caspase-3 and -7 were induced by UA treatment in LNCaP cell and not in PC3 and DU145 cells (Fig. 4.4C). On the other hand, digoxin down-regulated expressions of key cell cycle genes in all three cell lines tested (Fig. 4.4D). Together, these data suggest that the anti-cancer PCa cell growth effects of UA is through its inhibition of RORγ function in control of AR-signaling pathway.

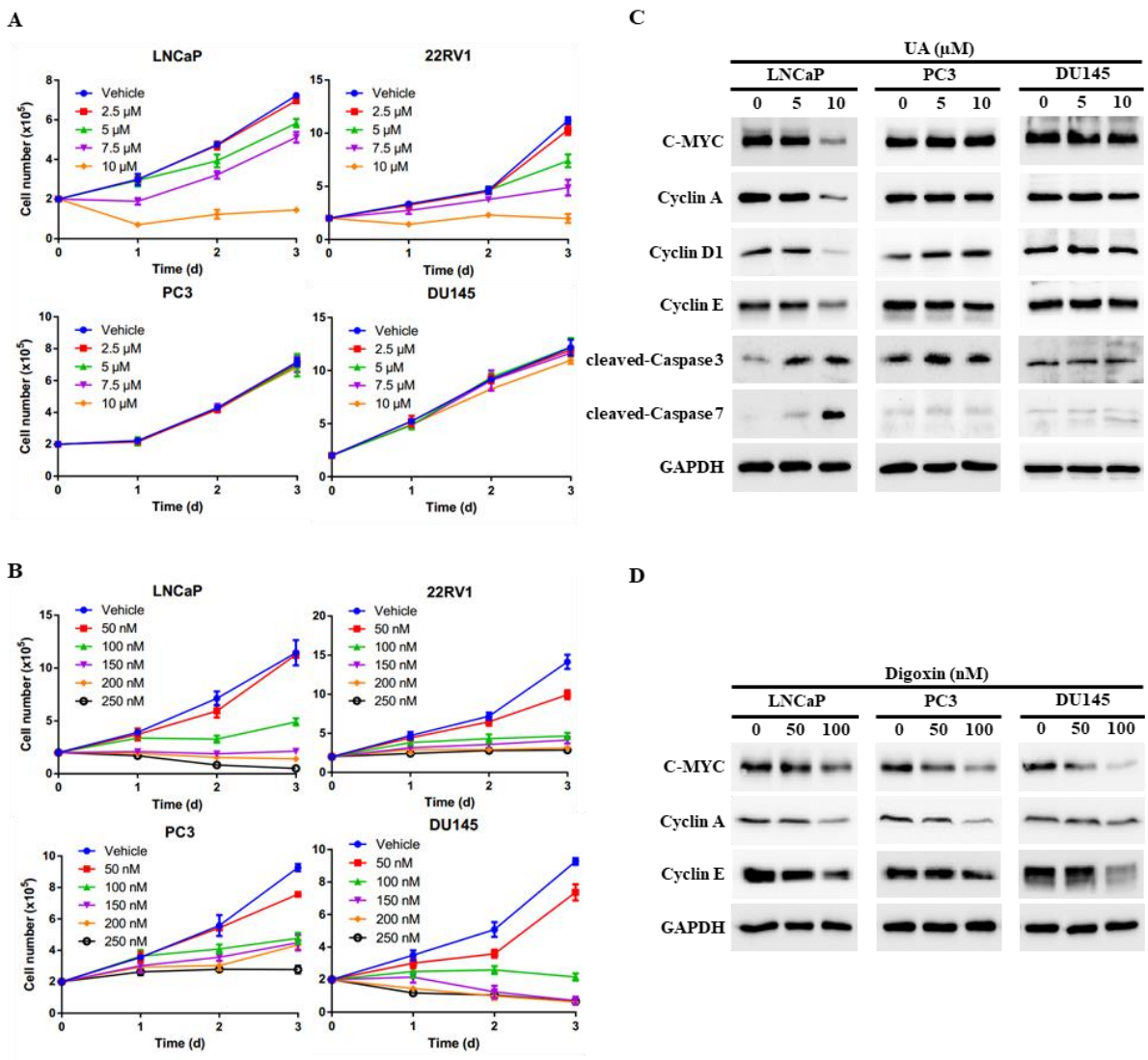

Figure 4.4 UA exhibits stronger inhibitory activity in AR-positive PCa compared with AR-negative PCa

A-B. AR-positive PCa cells (LNCaP and 22RV1) and AR-negative PCa cells (PC3 and DU145) were treated by UA (**A**) or digoxin (**B**) at indicated concentrations. One, two and three days later, viable cells were counted. **C-D**. AR-positive PCa cell (LNCaP) and AR-negative PCa cells (PC3 and DU145) were treated by UA (**A**) or digoxin (**B**) at indicated concentrations. Two days later, cells were harvested for western blotting analysis of indicated proteins. Representative blots, n = 3.

4.4.5 UA not digoxin alters RORγ-controlled expression of cell cycle and apoptosis genes

To further elucidate the effects of UA on RORγ function in cancer cells, we analyzed RNA-seq data we obtained from TNBC cells treated by UA and our ChIP-seq data of RORγ genome occupancy in TNBC cells [20]. Our analysis revealed a strong overlap between genes with

altered expression by UA and genes that displayed RORγ ChIP-seq peaks. Specifically, 30.7% of genes down-regulated by UA displayed reduced RORγ ChIP-seq peaks after the antagonist XY018 treatment (Fig. 4.5A), whereas 33.3% of genes up-regulated by UA had increased RORγ ChIP-seq peaks after XY018 treatment (Fig. 4.5B). Gene ontology (GO) analysis of genes with both reduced expression and ChIP-seq peaks revealed that DNA replication and cell proliferation/division were among the most enriched programs with representative genes such as POLA1, MCM6 and MKI67 (Fig. 4.5C and Fig. 4.5E, top panels). On the other hand, apoptotic process was one of the most enriched programs among genes being both UA-increased in expressions and XY018-increased in ChIP-seq peaks (e.g. BNIP3, BMF and BIK) (Fig. 4.5D and Fig. 4.5E, bottom panels). Our further RNA-seq and qRT-PCR analyses showed that the mRNA expression of RORγ direct target genes involved in cell cycle/cell proliferation was reduced by both XY018 and UA, while genes involved in apoptosis were induced. In contrast, digoxin displayed either little or no effect at 24 hours, or mostly activating effects at 48 hours on those genes particularly those of the cell cycle/proliferation (Fig. 4.5F). Together, the results suggest that like antagonist XY018, UA alters the expression of genes that are direct targets of RORγ whereas the effects of digoxin on gene expression in TNBC cells do not support the notion that digoxin acts through RORγ.

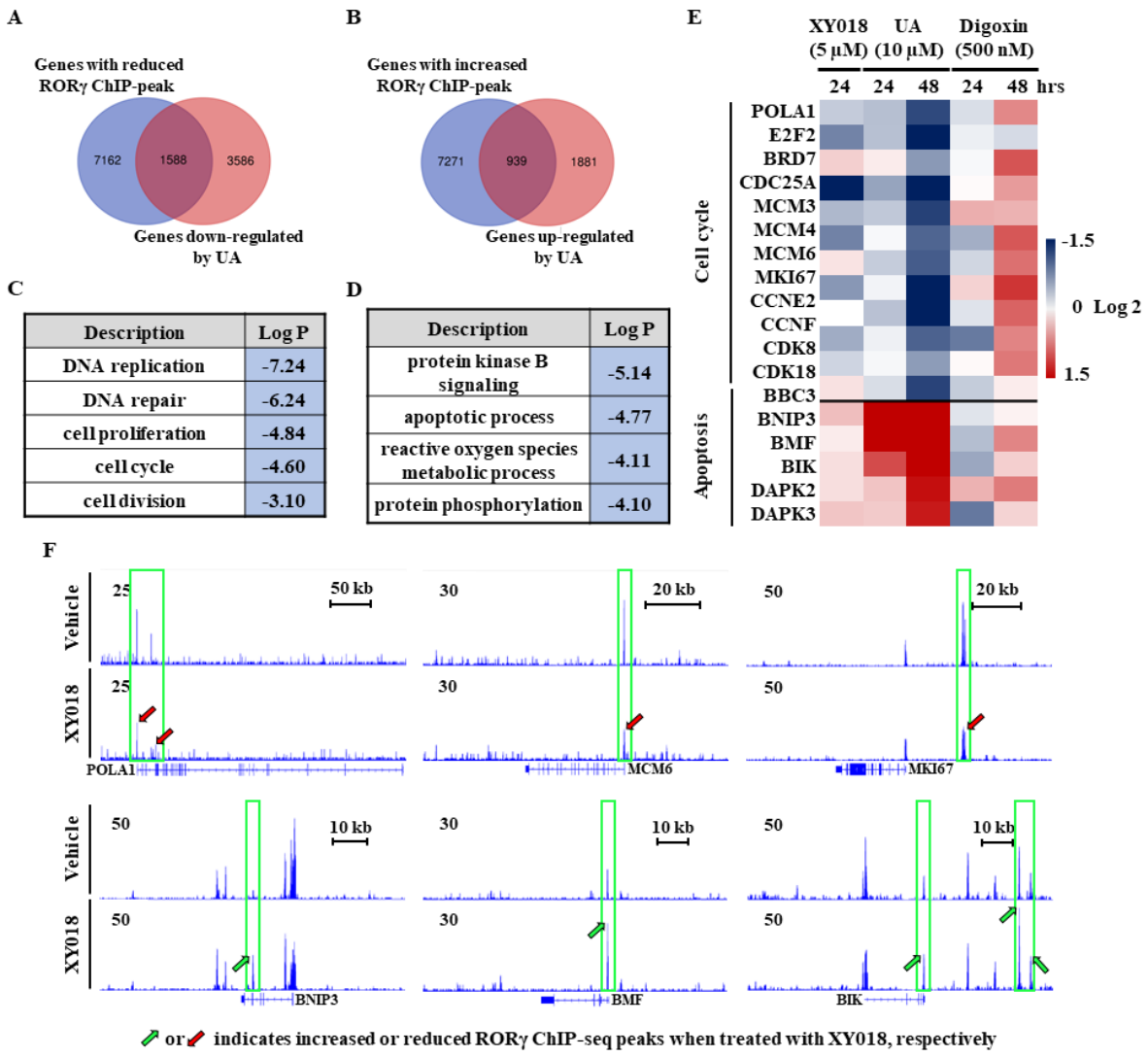

Figure 4.5 Expression of RORγ-controlled cell cycle and apoptosis genes was altered by UA but not digoxin in TNBC cells

A-B. Venn diagram of number of genes with altered RORγ ChIP-peaks overlapped with genes altered by UA treatment. **C-D.** Gene ontology analysis of genes with both reduced **(C)** or increased **(D)** expression and RORγ ChIP-peaks as shown in **A** or **B**. **E.** Heat map display of fold changes (in log 2) of RORγ direct target cell cycle and apoptosis gene mRNA in HCC70 cells analyzed by RNA-seq (UA) or qPCR (XY018 and digoxin) at indicated condition. **F.** ChIP-seq signal visualization of RORγ at representative cell cycle and apoptosis genes in HCC70 cells treated with 2.5 μM of XY018 or vehicle for 24h.

4.4.6 UA not digoxin suppresses RORγ-mediated cholesterol biosynthesis gene program

Our previous study demonstrated that RORγ directly controls cholesterol biosynthesis gene expression in TNBC cells [20]. To further validate that UA but not digoxin targets RORγ-mediated signaling in TNBC, we analyzed the effects of UA and digoxin on cholesterol biosynthesis gene expression in HCC70 cells. Our RNA-seq and qRT-PCR analysis showed that UA treatment down-regulated the expression of the majority of cholesterol-biosynthesis genes including those of the rate-limiting or key enzymes such as HMGCS1, HMGCR, MVK and SQLE. In contrast, digoxin up-regulated their expression (Fig. 4.6A). Indeed, GSEA showed that cholesterol-biosynthesis gene programs were significantly disrupted by UA treatment after 24 hours of treatment (Fig. 4.6B). Western blotting analysis also confirmed that protein expression of some of the key cholesterol-biosynthesis enzymes such as HMGCS1, HMGCR and SQLE were potently inhibited by UA treatment while remained unchanged when treated with digoxin (Fig. 4.6C). Together with the other data in this study (Fig. 4.2 and 4.5), these results strongly suggest that the anti-tumor effects of UA but not digoxin in TNBC is at least partially through its inhibition of RORγ-mediated signaling.

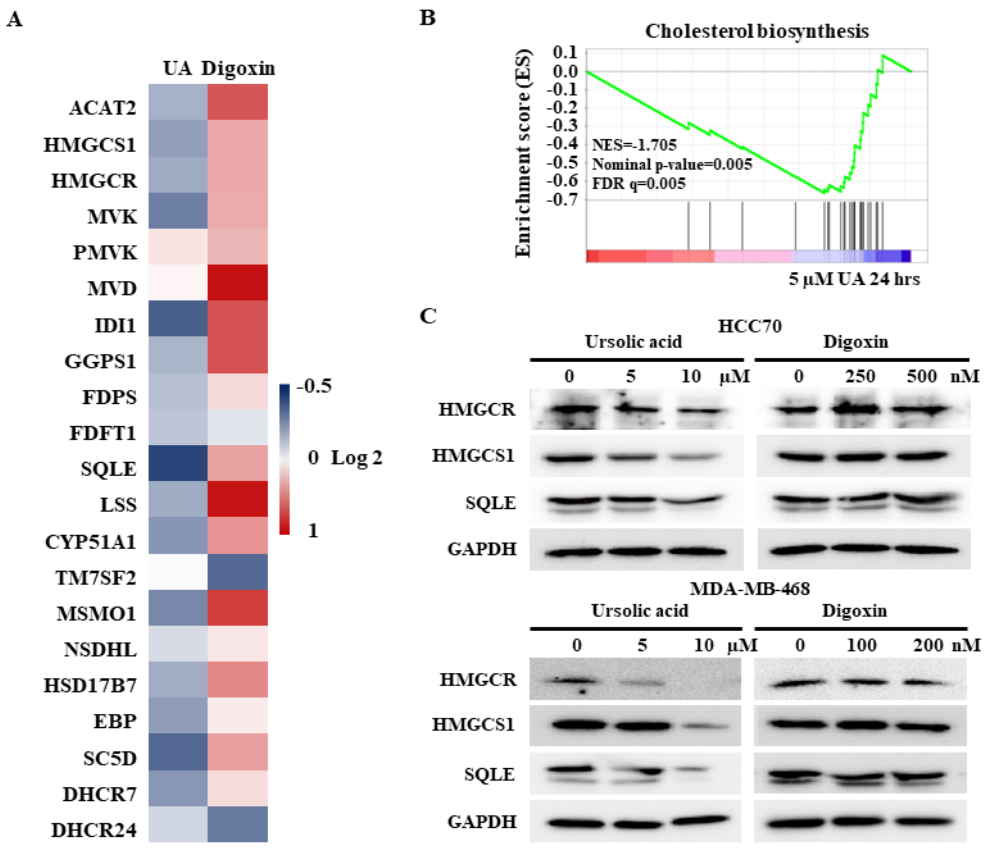

Figure 4.6 Expressions of cholesterol biosynthesis genes were altered by ursolic acid in TNBC cells

A. Heat map display of fold changes (in log 2) in mRNA expression of 21 cholesterol-biosynthesis genes in HCC70 cells treated by 5 μM UA (RNA-seq) or 250 nM digoxin (qPCR) for 24 hours. **B.** GSEA of genes involved in cholesterol-biosynthesis pathway in HCC70 cell treated with 5 μM UA for 24 hours. NES, normalized enrichment score. FDR, false-discovery rate. **C.** Immunoblotting of HMGCR, HMGCS1, SQLE and GAPDH in HCC70 and MDA-MB-468 cells treated with UA or digoxin at indicated concentration for 48 hours. Representative blots, n = 3.

4.5 Discussion

Digoxin, also known as digitalis, is prescribed to treat heart conditions. Its well-known mechanism of action (MOA) is its inhibition of Na+/K+ ATPase in the myocardium [29]. Recent studies also demonstrated that digoxin can modulate several cellular signaling pathways including NF-κB [30] or EGFR-STAT3 signaling [31]. In an early search for RORγ ligands, digoxin was claimed as a natural RORγt inverse agonist/antagonist in Th17 immune cells [14].

Several later studies also showed that 5-10 μM of digoxin can suppress RORγt-mediated Th17 differentiation and IL-17 production [32,33,34]. However, results from our study using cancer cell models do not support the conclusion that digoxin, at concentrations that are comparable to its use as an anti-cancer agent, can act as RORγ antagonist. We demonstrated in PCa cells that, at sub-micromolar concentrations, digoxin has modest but significant activating effects on the expression of RORγ direct target gene AR and AR signaling genes. Likewise, in TNBC cells, the expression of cell cycle and cholesterol biosynthesis gene programs that are directly activated by RORγ are also induced by digoxin treatment. These results strongly argue against the notion that digoxin can act as an RORγ antagonist in cancer cells. In fact, our finding is consistent with a recent study showing that digoxin can act as an RORγ agonist and induce RORγt-dependent transcription at sub-micromolar concentrations in the cells examined [35]. However, considering that digoxin can target Na+/K+ ATPase [29] and regulate other pathways [30,31], further studies are needed to determine whether any of the effects of digoxin in the cancer cells is through RORγ or other pathways.

Unlike digoxin, in this study we found that UA strongly inhibits the expression of AR, a direct target of RORγ in PCa cells. In our RNA-seq analysis, we revealed that UA can inhibit the expression of AR signaling genes that are positively regulated by AR [28], consistent with our previous finding that RORγ stimulates the AR signaling gene program. In addition, we found that the strong anti-proliferation effect of UA can be observed only in AR-positive PCa cells but not AR-negative cells. These data together strongly suggested that the effects by UA treatment on AR expression and signaling is likely through its inhibition of RORγ function in the PCa cells. Several studies showed that UA can inhibit cancer cell and tumor growth by interfering with cell cycle, proliferation, apoptosis, inflammation, angiogenesis, and metastasis [36,37,38]. However, there has been no evidence showing that UA can affect AR expression and/or AR signaling, which is the key driver of PCa development and progression. Of note,

inhibition of AR by UA can be observed as low as 10 μM of UA, while the effects by UA on the other processes were reported at much higher concentration (20-50 μM) of UA [3,39,40,41]. Thus, it is possible that in PCa cells, disruption of AR signaling through inhibition of RORγ is the primary MOA of UA.

UA has been shown to display anti-growth effects in cells of different cancers, including prostate cancer, breast cancer, lung cancer, colorectal cancer, and pancreatic cancer [38,42]. However, the direct target of UA in cancer cells remains unclear. Our study here demonstrated in PCa and TNBC cells that UA inhibits the expression of gene programs such as AR signaling and cholesterol biosynthesis that are directly controlled by RORγ. Our integrated analysis of ChIP-seq and ATAC-seq data also revealed that gene programs affected by UA correlate closely with the ones directly controlled by RORγ in the cancer cells. Together, these results strongly support the conclusion that in the cancer cells, UA can act as an antagonist to RORγ. Although previous studies showed that UA can act as RORγt inhibitor in immune cells such as Th17 cells [13,43], our study here provides for the first-time evidence that UA displays RORγ antagonism activity in cancer cells. Similar to UA, recent studies identified additional natural compounds such as elaiophylin [19] and N-hydroxyapiosporamide [22] as RORγ antagonists. Despite their structural differences, these natural compounds share similar inhibitory effects on the gene programs controlled by RORγ in the cancer cells and tumors. Given that natural agents often possess effects on multiple cellular and molecular targets, it is critical that thorough investigations are performed to better understand their MOA in order to further develop them for effective clinical use.

4.6 Acknowledgements

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4.7 Supplementary Materials

Supplementary Table 4.1

Primers for human qRT-PCR	
GAPDH-F	GCAGGGATGATGTTCTG
GAPDH-R	GTATGACAACAGCCTCAA
POLA1-F	GCTTCACCTCTGACCTTTAC
POLA1-R	GTACAGGGACTTGTCAGAATAC
E2F2-F	CAGCTACTGCTACCTACTACA
E2F2-R	CATCCACTCTGATGCACTTC
BRD7-F	CTTGATGAGACCACCAGATTG
BRD7-R	CGAACTCCATACGTGCTTAC
CDC25A-F	CTCCTCTCGTCATGAGAACTA
CDC25A-R	GGAAGATGCCAGGGATAAAG
MCM3-F	CCTATGCCAAGCAGTATGAG
MCM3-R	GGGTGGTGAGATCAGAATAAC
MCM4-F	CCTCTGGCTAAAGAAGAAGAAA
MCM4-R	CAGGGTAACGGTCAAAGAAG
MCM6-F	GGAAACACCTGATGTCAATCTA
MCM6-R	CCTCTTCTTCTTCCACCTTTC
MKI67-F	GTCACACCGAGGAATTAGTG
MKI67-R	CTTCACCTACTGATGGTTTAGG
CCNE2-F	CCTCCATTGAAGTGGTTAAGA
CCNE2-R	CCTCCAGCATAGCCAAATAG
CCNF-F	TCACCGGATTCTCCTATGAA
CCNF-R	CTGTCTTGTGTCACTCCTAATG
CDK8-F	CGGAAGAAGAACCTGATGAC
CDK8-R	GTGGATTGGAACGCTGATAG
CDK18-F	CCTATGCCACAGTCTTCAAA
CDK18-R	CACTGTCCAGGTACTCAAAC
BBC3-F	ATCCCATTGCATAGGTTTAGAG
BBC3-R	CTACAGCAGCGCATATACAG
BNIP3-F	CTCAGATTGGATATGGGATTGG
BNIP3-R	CAAATGAGAGAGCAGCAGAG
BMF-F	CAGTTTCCCAGCAGTCTTG
BMF-R	GTTCCTGTTCTCTTCTCCATTC
BIK-F	GGAGGACTTCGATTCTTTGG
BIK-R	TCTCCTTAAGTGTGGTGAAAC
DAPK2-F	GCTGACATGTGGAGCATAG
DAPK2-R	GTTTCCGGGTCTCTTTAACC
DAPK3-F	ATGCTGCTGGACAAGAAC
DAPK3-R	CTCAGGAGGATATAGGTGATGA
ACAT2-F	GTGCTGCAGCTGTCGTTCTTAT
ACAT2-R	CTTCCAGTGACCAACCTGCTTT
HMGCS1-F	CTTTCGTGGCTCACTCCCTTTC

HMGCS1-R	AGGGCAACAATTCCCACATCTT
HMGCR-F	GCACCAAGAAGACAGCCTGAATAG
HMGCR-R	TCTGAGGAGTCTGCATGGAAAGA
MVK-F	CCTCAGCTTACCCAACATTGGTATC
MVK-R	TCCGGCAGATGGACAGGTATAA
PMVK-F	AGTGGTTTCGGGAGGCCTAT
PMVK-R	TCAGGTTCTCCAACTGCTCCT
MVD-F	TGGCATCGGTGAACAACTTCC
MVD-R	CCCATCTGCCACTCCACAAAG
IDI1-F	CACACCCTGGATATGTGTTCTGTTT
IDI1-R	TCTGCAAGTGCTCCGGAAATG
GGPS1-F	GGCAGTTCCAAGCCAGTTTCTA
GGPS1-R	CCTCCCAAAGTGCTGGGATTAC
FDPS-F	GCCAAGGAAACAGGATGCTGATAG
FDPS-R	AGCTTCAGCAGGCGGTAGATA
FDFT1-F	GGTCCCGCTGTTACACAACTTT
FDFT1-R	GCCATCCCAATGCCCATTCT
SQLE-F	CCATGCTCCACTGACTGTTGTT
SQLE-R	AGATGAGAACTGGACTCGGGTTAG
LSS-F	GCGAGGAGCGGCGTTATTT
LSS-R	TGTAGGAGATGGCACAGGACTT
CYP51A1-F	TTGGCTGCCTTTGCCTAGTT
CYP51A1-R	GCTGCCCTGCCAAGAGTAAT
TM7SF2-F	CACCCTCACCGCTTTCATCTT
TM7SF2-R	CGGGTCGCAGTTCACAGAAATA
SC4MOL-F	CCTCCCAAAGTGCTGGGATTAC
SC4MOL-R	AAGTTCTTGGTGCCGGCTTT
NSDHL-F	GCCAGGAACGGCAAGATGAA
NSDHL-R	GCGAGACAGGAATGTCCAGAAAG
HSD17B7-F	CCACCACTGGCTTTGGAAGAA
HSD17B7-R	GCCTCCCAAAGTGCTGGAATTA
EBP-F	ACTGGCCTCAGCACCTAAGA
EBP-R	AACCCACACACTGCAAACCA
SC5D-F	GTTGCACCATCCCTGGTTTCT
SC5D-R	CTGCCCTCTGCAACTGATTTCT
DHCR7-F	CATTGACATCTGCCATGACC
DHCR7-R	ACAGGTCCTTCTGGTGGTTG
DHCR24-F	TGTTGCCTGAGCTTGATGAC
DHCR24-R	GACCAGGGTACGGCATAGAA

Supplementary Table 4.2

Antibody	Vendor	Catalog number
AR	Santa Cruz	SC-7305
AR-V7	Cell signaling	68492
Cleaved-Caspase 3	Cell signaling	9664
Cleaved-Caspase 7	Cell signaling	9491
C-MYC	Santa Cruz	SC-40
Cyclin A	Santa Cruz	SC-271682
Cyclin D1	Santa Cruz	SC-8396
Cyclin E	Santa Cruz	SC-247

HMGCR	Santa Cruz	SC-271595
HMGCS1	Santa Cruz	SC-166763
RORγ	Invitrogen	14-6988-82
SQLE	Santa Cruz	SC-271651
GAPDH	Cell signaling	2118

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Chapter 5

Conclusion

NR family member RORγ and its immune cell isoform RORγt play a crucial role in control of circadian rhythm, metabolism, Th17 cell differentiation and autoimmune disorders. They emerged recently as a potential therapeutic target for treatment of autoimmune disease and several types of cancer [1,2,3,4,5,6,7,8]. Consequently, many small molecules targeting RORγ/ RORγt have been developed, demonstrating their effectiveness for cancer or auto-immune disease treatment in pre-clinical studies and, notably, in ongoing clinical trials. As mentioned previously, in contrast to treatment for auto-immune disease, the exploration of RORγ as a target for cancer therapeutics commenced only in recent years. The seminal study, published in 2016, revealed that RORγ directly regulates AR gene expression and AR signaling in CRPC cells and tumors [1]. The small molecule inhibitor, XY018, displayed potent inhibitory effects on xenograft and PDX tumor growth. Subsequent studies have further elucidated the pivotal role of RORγ in tumor development and progression across various cancer types, including breast, pancreatic, and lung cancer [2,3,6]. Importantly, all of the referenced studies utilized small molecule inhibitors, either synthetic or natural, underscoring the significance of RORγ small molecule inhibitors as indispensable tools for studying RORγ function in cancer research. These findings also emphasize the potential for further development of these inhibitors as effective therapeutics for cancer.

Recent advancements in synthetic small molecule inhibitors targeting RORγ have expanded our understanding of their potential in cancer treatment. Prominent among these inhibitors are GSK805, SR2211, XY018/XY101/XY123, and betulinic acid derivatives [1,2,9,10,11]. While the XY compounds and betulinic acid derivatives were initially reported as RORγ inhibitors in cancer cells, GSK805 and SR2211 were developed to inhibit RORγt function in immune cells. In my study (Chapter 3), I conducted a comparative analysis of several synthetic RORγ

inhibitors to evaluate their efficacy in inhibiting RORγ function in triple-negative breast cancer (TNBC) cells. This investigation revealed distinct context-specific activities of these compounds between cancer cells and immune cells. Similarly, I examined the activities of natural RORγ antagonists, such as digoxin and UA, in disrupting RORγ function in prostate cancer cells (Chapter 4). Only UA displayed inhibitory effects on tumor growth by disrupting RORγ function, highlighting the context-specific modulation of RORγ or RORγt functions of different RORγ inhibitors. The results also suggested that the distinct activities of various RORγ antagonists are, in part, influenced by their ability to access RORγ-associated regulatory complexes or alter chromatin structure.

The observed context-specific activities of RORγ inhibitors offer promising avenues for future development in cancer therapy. While recent studies have implicated RORγ in promoting cancer progression in prostate, breast, lung, and pancreatic cancers, its isoform, RORγt, has long been recognized for its crucial role in immune system development, particularly in Th17 cell differentiation. It remains plausible that RORγ antagonists targeting cancer cells may also affect normal RORγt function in immune cells. Therefore, the promising inhibitory effects of these inhibitors on tumor growth and progression may be mitigated by potential impacts on immune system function. Indeed, compounds like XY018, GSK805, SR2211, and UA exhibit moderate levels of antagonistic effects in immune cells, suggesting the need to minimize antagonistic effects on immune cells while preserving efficacy in cancer cells through chemical structure modifications.

Notably, RORγt agonists have been developed and examined in combination with anti-PD-1 or anti- CTLA-4 treatments as part of immune-based therapeutic approaches [12,13]. Indeed, a RORγt agonists, LYC-55716, have emerged and are undergoing clinical investigations for cancer treatment [14]. Finding from my studies described here that RORγ-targeting compounds display tissue-dependent, context-specific activities, indicating the potential for development

of unique RORγ-targeting compounds that act as agonists in immune cells while also displaying potent antagonistic properties in cancer cells. Such unique RORγ-targeting compounds would disrupt RORγ-mediated transcriptional programs in cancer cells, inhibiting cancer development and progression, while simultaneously promoting Th17 and other immune cell differentiation to enhance immune system's anti-cancer activity. This dual mechanism holds greater promise for effective treatment of the different cancer types where RORγ plays a crucial role in the disease progression.

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