DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME INSTITUTE

CO-TARGETING OF EZH2 AND RETINOIC ACID SIGNALING IN BLADDER CANCER

GİZEM ÖZGÜN

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ABBREVIATIONS

AKR1C2 : Aldo-Keto Reductase Family 1 Member C2
ARID1A : AT-Rich Interaction Domain 1A
ATCC : The American Type Culture Collection
ATF : Activating Transcription Factor
BAM : Binary Alignment Map
BCA : Bicinchoninic acid
BSA : Bovine Serum Albumin
C/EBPβ : CCAAT Enhancer Binding Protein Beta
CASP4 : Caspase 4
CDH1 : Cadherin 1
CDKN1C : Cyclin-Dependent Kinase Inhibitor 1C
cDNA : Complementary DNA
ChIP : Chromatin Immunoprecipitation
ChIP-seq : Chromatin Immunoprecipitation Sequencing
CHOP : C/EBP Homologous Protein
CoFeDR : Downregulated by fenretinide-only or combination treatments
CoFeUR : Upregulated by fenretinide-only or combination treatments
CoSDR : Strongly downregulated by combination treatment
DMEM : Dulbecco's Modified Eagle Medium
DMSO : Dimethyl sulfoxide
DNMT DNA methyltransferases
DSMZ : German Collection of Microorganisms and Cell Cultures
EP300 : E1A-associated Protein p300
ER : Endoplasmic reticulum
EZH2 : Enhancer of Zeste 2
FBS : Fetal Bovine Serum
FDA : The Food and Drug Administration
FeUR : Upregulated by fenretinide treatment

FITC : Fluorescein isothiocyanate
FOXA1 : Forkhead Box A1
FPKM : Fragments per kilobase of transcript per million mapped reads
GADD45A : Growth Arrest and DNA Damage Inducible Alpha
GATA4 : GATA Binding Protein 4
GO : Gene Ontology
GskUR : Upregulated by GSK-126 treatment
H3K27me3 : Ttrimethylation of lysine 27 on histone H3
HAT Histone Acetyl Transferases

HDAC Histone Deacetylases
HMT Histone Methyl Transferases
HSPB8 : Heat Shock Protein Family B Member 8
IC : Inhibitory concentration
IGFBP3 : Insulin-like growth factor-binding protein 3
KDM Histone Lysine Demethylase
KMT : Lysine N-methyltransferase
log2 FC : Log 2 fold change
MEIS3 : Meis Homeobox 3
MIBC : Muscle-Invasive Bladder Cancer
NCBI : The National Center for Biotechnology Information
NDRG1 : N-myc Downstream-Regulated Gene 1
NFE2 : Nuclear Factor, Erythroid 2
NMIBC : Non-Muscle-Invasive Bladder Cancer
PBS : Phosphate Buffered Saline
PI : Propidium iodide
PIC : Protease inhibitor cocktail
PRC2 : Polycomb Repressive Complex 2
PTGS2 : Prostaglandin-Endoperoxide Synthase 2
RA : Retinoic Acid
RAR : Retinoic Acid Receptor
RNA : Ribonucleic acid
RNA-seq : RNA sequencing
RT-qPCR : The Quantitative Real-Time Reverse Transcription Polymerase Chain
RXR : Retinoid X Receptor
SAM : S-adenosylmethionine

SERPINE2 : Serpin Peptidase Inhibitor, Clade E, Member 2
SET : Su(var)3-9, Enhancer of Zeste, and Trithorax
STAT3 : Signal Transducers and Activators of Transcription 3
SWI/SNF : Switching/Sucrose Non-Fermenting
TBST : Tris-buffered Saline and Tween 20
TCA : Trichloroacetic acid TCGA : The Cancer Genome Atlas tRNA : Transfer RNA TSS : Transcription Start Site
UPK1A : Uroplakin 1A
UPR : Unfolded Protein Response
USF : Upstream Transcription Factor

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CO-TARGETING OF EZH2 AND RETINOIC ACID SIGNALING IN BLADDER CANCER

Gizem Özgün, Izmir International Biomedicine and Genome Institute

ABSTRACT

The prevalence of an excessive amount of mutations in bladder cancer, particularly in chromatin regulatory genes that oppose Polycomb-mediated repression, and the role of EZH2 overexpression contributing to the worse prognosis highlight the significance of targeting EZH2 in bladder cancer. Furthermore, the crucial role of the retinoic acid (RA) signaling pathway in the development and homeostasis of urothelium and the anti-oncogenic features of retinoids are well established. Thus, our aim is to simultaneously target the RA signaling pathway and EZH2 in bladder cancer to potentiate the therapeutic response and to elucidate the molecular mechanisms underlying this strategy. In this study, we reported that coordinated targeting of the RA signaling pathway and EZH2 elicited potent cytotoxic effects on bladder cancer cells by synergistic suppression of proliferation linked with induced apoptosis and cell cycle arrest in an orchestrated manner. The combined therapy resulted in the regulation of gene expression associated with the anti-oncogenic profile, as reflected by the activation of a subset of marker genes linked with apoptosis, epithelial phenotype, and urothelial differentiation. The transcriptional regulators C/EBPβ and CHOP were identified as key mediators of anti-oncogenic transcriptional reprogramming by stimulating the endoplasmic reticulum stress response. A molecular mechanism by which EZH2 sustains repression of a subset of genes primarily involved in unfolded protein response and certain metabolic processes was depicted. Overall, our findings emphasize the benefit of co-targeting the EZH2/RA pathway in bladder cancer and encourage the design of clinical studies to investigate the efficacy of this combination strategy.

Keywords

Bladder cancer, combination therapies, epigenetic drugs, EZH2 inhibitors, retinoids

MESANE KANSERİNDE EZH2 VE RETİNOİK ASİT SİNYALİNİN BİRLİKTE HEDEFLENMESİ

Gizem Özgün, İzmir Uluslararası Biyotıp ve Genom Enstitisü

ÖZET

Mesane kanserinin özellikle Polycomb-aracılı baskılamayı tersine çeviren kromatin düzenleyici genlerde oldukça yüksek oranda mutasyon barındırması ve EZH2'nin artan ekspresyonunun kötü prognoza katkıda bulunan rolü, mesane kanserinde EZH2'yi hedeflemenin önemini vurgulamaktadır. Ayrıca, retinoik asit (RA) sinyal yolağının ürotelyum gelişimi ve homeostazındaki kritik rolü ve retinoidlerin anti-onkojenik etkileri iyi bilinmektedir. Bu nedenle amacımız, mesane kanserinde terapötik yanıtı artırmak için RA sinyal yolağı ve EZH2'yi birlikte hedeflemek ve bu stratejinin temelini oluşturan moleküler mekanizmaları aydınlanmaktır. Bu çalışmada, mesane kanserinde EZH2 ve RA sinyal yolağının birlikte hedeflenmesinin apoptozu ve hücre döngüsü durmasını indükleyerek hücre çoğalmasının baskılanması üzerinde güçlü bir sinerjik etki ortaya çıkardığı bildirilmiştir. Kombine tedavi, apoptoz, epitelyal fenotip ve ürotelyal farklılaşma ile bağlantılı işaretleyici bir gen alt kümesinin aktivasyonunu artırarak gen düzenlenmesi açısından anti-onkojenik bir profil yansıtmıştır. Anti-onkojenik transkripsiyonel yeniden programlamanın ana aracıları olarak endoplazmik retikulum stres tepkisini uyaran C/EBPβ ve CHOP transkripsiyonel faktörleri tanımlanmıştır. EZH2'nin, esas olarak katlanmamış protein yanıtı ve belirli metabolik süreçlerde yer alan birtakım gen alt kümelerinin baskılanmasını sürdürdüğü bir moleküler mekanizma betimlenmiştir. Genel olarak, bulgularımız mesane kanserlerinde EZH2/RA yolağını birlikte hedeflemenin yararını vurgulamakta ve bu kombinasyon stratejisinin etkinliğini araştırmaya yönelik klinik çalışmaların tasarımını teşvik etmektedir.

Anahtar Kelimeler

Mesane kanseri, kombinasyon tedavileri, epigenetik ilaçlar, EZH2 inhibitörleri, retinoidler

1 RATIONALE AND SCOPE OF THE THESIS

As a signature of the disease, bladder cancer harbors a highly enriched mutational load, notable in chromatin modulatory genes counteracting Polycomb-mediated repression. Besides,

overexpression of EZH2 is associated with a worse prognosis for bladder cancer. Consequently, EZH2 is portrayed as a potential therapeutic target for bladder cancer. Furthermore, Polycomb Repressive Complex 2 (PRC2) has a co-repressor function modulating the expression of retinoids, which act as chemopreventive agents.

The striking heterogeneity of bladder cancer suggests that targeting a single molecular pathway is unlikely to resolve the underlying mechanisms of molecular and cellular complexity. Considering that both EZH2 and the retinoic acid pathway play, in principle, important roles in bladder cancer and have distinct modes of action, we set out to investigate the theory that simultaneous targeting of the retinoic acid pathway activation and EZH2 inhibition could be an effective strategy in bladder cancer therapy.

The major aims of this project were:

To ascertain the efficacy of coordinated targeting of the retinoic acid pathway and EZH2 in bladder cancer,

To elucidate the alterations in the phenotypic, transcriptomic, and epigenetic landscape of bladder cancer cells linked to co-treatment with a retinoid and a potent EZH2 inhibitor

To enlighten the major molecular mechanisms underlying the interactions of this co-targeting strategy

2 LITERATURE REVIEW

2.1 Bladder Cancer

Bladder cancer is one of the most frequently diagnosed tumors globally, as reported by global cancer data [1]. Bladder cancer is a genetically and epigenetically heterogeneous disease at the genomic, transcriptional, and cellular levels and consists of a high number of genetic alterations linked to functionally different sub-groups [2]. Bladder cancer frequently develops from the epithelium of the bladder and is described as urothelial carcinoma. Global transcriptional profiling has elicited the identification of different subtypes of the disease bearing divergent pathological features and distinctive molecular characteristics. Non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) represent the two major subgroups of urothelial carcinoma based on clinical stage, each having a unique clinical prognosis and treatment options [3]. NMIBC is an early-stage malignancy that has not advanced beyond the epithelial layer, represents approximately 75% of primary diagnoses, and is specified by frequent recurrence and a relatively low mortality risk [4]. MIBC is an aggressive form of the disease in which the tumor has invaded the detrusor muscle, is more vulnerable to metastasis, and contributes to high morbidity and mortality [5].

Neoadjuvant chemotherapy based on cisplatin coupled with radical cystectomy is the current standard therapy regimen for MIBC [6]. Nevertheless, most of the patients only experience a temporary benefit from these regimens, and estimates of disease-specific survival indicate that chemotherapy adds only minor benefits to definitive surgery [7]. The highly proliferative, multifocal, diverse mutations harboring, and metastatic characteristics of the disease underlie the poor prognosis of MIBC patients. Finding innovative targeted therapies with a wide therapeutic range has been significantly hampered by these disease features [2]. Recently, molecular profiling has led to the discovery of potential biomarkers to improve the prognosis for MIBC patients. Nevertheless, the classification of molecular characterization has not been transformed into efficient targeted therapeutics yet, and drug innovation in that field has been stagnant. Overall, recurrence and frequent resistance to conventional and targeted therapies emphasize the need for developing more potent treatment options for these patients. By focusing on the discovery of novel therapeutic

targets, future investigations could open new avenues that might revolutionize the paradigm of bladder cancer treatment.

2.2 Epigenetic Dysregulations of Bladder Cancer

Progress in tumor genomic analysis has uncovered prominent bladder cancer-specific mutational signatures. As a key feature of the disease, bladder cancer harbors a particularly high frequency of genetic alterations in chromatin remodeling genes encoding proteins, including histone demethylases (KDM6A), histone methyltransferases (KMT2C and KMT2D), histone acetyltransferases (CREBBP and EP300), and the SWI/SNF chromatin remodeling complex (ARID1A) [8–11] (Figure 1). Remarkably, most of these chromatin modifiers oppose Polycombmediated repression by participating in active chromatin organization. Additionally, integrated network analysis revealed that those mutations had a significant influence on the levels of numerous transcription factors and pathways linked to cancer [9]. The characteristic of bladder cancer, which harbors excessive modifications in epigenetic machinery, produces novel therapy options for this disease. In line with this, genomic sequencing has uncovered that the loss of KDM6A, which increases EZH2-mediated transcriptional repression, makes MIBC more susceptible to EZH2 inhibitors [12].

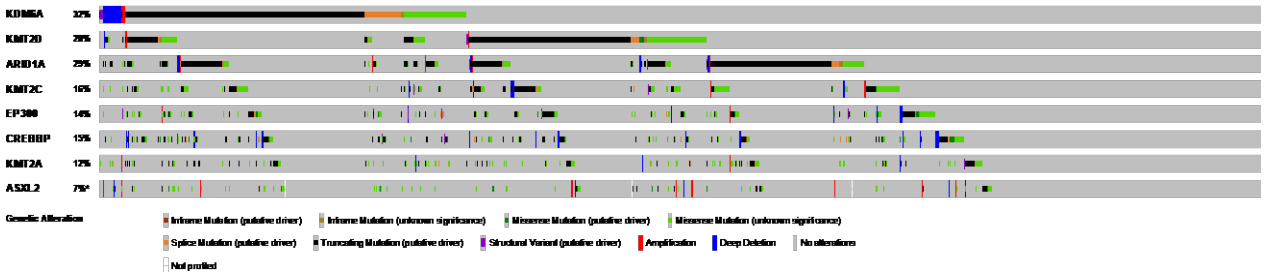


Figure 1. Primary epigenetic modifications in human bladder cancer

Whole-transcriptome profiling of patient samples revealed frequently mutated genes in bladder cancer. The data were obtained from the three studies (MSK, Cell Reports 2022, MSK, TCGA, 2020, and TCGA, Cell 2017) in CBioPortal [13] and reflect the frequency of mutated genes in bladder cancer patient samples (2548 samples/2132 patients) and the percentages of the various types of modifications per gene.

2.3 Epigenetic Drugs

The core of epigenetics is a broad range of covalent modifications applied to histone proteins and nucleic acids that work in concert to control chromatin organization and gene expression [14]. Cancer cells possess profoundly disrupted epigenetic patterns. Epigenetic dysregulation has primary functions in tumor formation, tumor progression, and the acquisition of treatment resistance and can affect all of the identified hallmarks of cancer [15]. Therefore, different strategies have been developed to generate epigenetic drugs, particularly targeting the enzymes that govern the epigenetic control of genome function.

Epigenetic modifiers are identified in four categories: the "writers," which transfer specific marks to DNA or the core histones H2A, H2B, H3, and H4; the "readers," which identify these marks; the "erasers," which eliminate them; and the "shapers," which reorganize chromatin or facilitate exchange of histones. Proteins representing the first three categories are the focus of current epigenetic therapies. The first category of epigenetic drugs targets writers such as DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), and histone methyltransferases (HMTs). Another class of epigenetic drugs that target erasers includes DNA-demethylating enzymes, histone deacetylases (HDACs), and histone demethylases (KDMs). A different class of epigenetic drugs represents drugs that target proteins that recognize methyl-CpGs and modified histones (Figure 2). Additionally, epigenetic therapeutic studies that target chromatin remodeling elements and histone chaperones are progressing [16].

Epigenetic dysregulation underlies both solid and hematological malignancies and can be targeted by diverse therapy strategies [16]. Nevertheless, up to this point, the effectiveness of epigenetic drugs has primarily been restricted to hematological cancers. This could possibly be due, in part, to the fact that solid tumors frequently develop from highly differentiated cells with a reduced epigenetic reprogramming capacity. The first- and second-generation epigenetic drugs, which are mainly DNMT inhibitors or HDAC inhibitors, had limited clinical success in patients with solid tumors since they had been designed with a "one size fits all" concept. The development of particular third-generation epigenetic drugs employed in accordance with precision medicine

paradigms, representing a more tailored option for their targets, opens new horizons in the branch of epigenetic therapy [16]. For instance, tazemetostat, a potent inhibitor of the enhancer of zeste homologue 2 (EZH2), has shown promise in treating patients with EZH2-mutant hematological cancers and severely aggressive solid cancers that carry genetic alterations that affect the SWI/SNF chromatin remodeling complex [17–19].

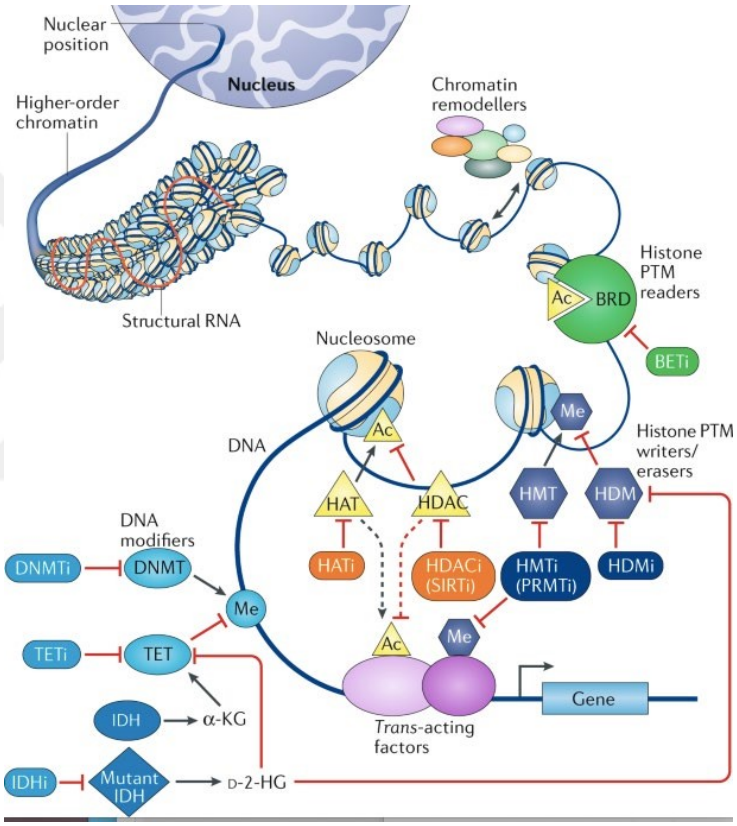


Figure 2. Targeting the epigenetic profile

Current epigenetic therapies focus on epigenetic modifier enzymes called writers, readers, and erasers. The first category of epigenetic drugs contains DNA-modifying enzyme inhibitors, such as DNA methyltransferases (DNMTs), which can methylate specific cytosines. Targeting gain-of-function mutationharboring isocitrate dehydrogenase (IDH) enzymes, which inhibit TET hydroxylases and cause abnormal DNA methylation, is the focus of another group of epigenetic drugs that influence DNA methylation. A different class of epigenetic drugs inhibit writers or erasers of post-translational modifications of histone arginine or lysine, such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs), histone deacetylases (HDACs), and histone demethylases (HDMs), which can also affect non-chromatin components. The readers of histone

post-translational modifications by proteins of the bromodomain and extra-terminal domain (BET) family, which interact with acetylated lysine residues of histones and other non-chromatin components, are inhibited by another class of epigenetic drugs (Adapted from [16], permission to use: license number: 5595380725255).

2.4 Combinational Therapies with Epigenetic Drugs

Through dynamic epigenetic modifications that provide them with a fitness advantage, cancers constantly change. By means of regulation of cancer cells or their microenvironment, this epigenetic plasticity is implicated in primary and acquired resistance to a variety of antitumor treatments. Reflecting this, epigenetic drugs are considered most successful when employed in combination with other therapies and provide compelling approaches for sensitizing cancer cells to a specific therapy and dynamically tackling acquired resistance mechanisms. Therefore, various trials have been designed to incorporate epigenetic drugs into the four pillars of anticancer therapy, including cytotoxic agents, hormone therapy, targeted therapies, and immunotherapies.

In preclinical trials investigating the effects of epigenetic drugs combined with chemotherapy, synergy is commonly acquired with DNA-damaging compounds [20]. Using HDAC inhibitors and DNMT inhibitors combined with genotoxic agents encourages global chromatin relaxation by making it easy to damage DNA, impairs DNA damage repair for genotoxic agents, and reverses resistance to cytotoxic drugs [21]. In preclinical trials, inhibiting HDAC, DNMT, EZH2, or BET was shown to enhance the antitumor efficiency of radiotherapy by hindering DNA damage repair and the cell cycle and boosting oxidative stress [22–25]. In ER-positive breast cancer models, HDAC inhibitors have been demonstrated to enhance the anticancer potency of hormone treatments or to overcome resistance mechanisms [26]. Due to the interaction of different epigenetic processes, combining various types of epigenetic drugs might represent an appealing anticancer therapeutic strategy. For instance, BET inhibition hinders H3K27 acetylation-mediated signaling and makes cancer cells more susceptible to EZH2 inhibitors [27]. Epigenetic drugs may also be used to modify antitumor immunity, considering specific DNA- or histone-modifying enzymes and histone chaperones improve the immune response of cancer cells [28].

2.5 Polycomb Group Proteins

One of the crucial processes governing cell fate decisions and maintaining cell identity during development is epigenetic control of gene expression. The evolutionarily conserved Polycomb group (PcG) and Trithorax group (TrxG) components function antagonistically to coordinate the expression of crucial genes in cell differentiation and developmental processes [29]. PcG-TrxG complexes preserve genomic integrity by altering DNA methylation, nucleosome location or composition, and covalent modifications of histone tails, thereby influencing chromatin organization and thus transcriptional status. PcG proteins typically repress transcription, as opposed to TrxG proteins, which function in active transcription states [30].

The PcG proteins possess a pivotal function in the fulfillment of cellular identity and cell fate decisions, serving as archetypal epigenetic modulators that promote the pursuit of cell-specific transcriptional programs [31]. They operate within multiprotein structures identified as Polycomb repressive complexes (PRCs), which cause modifications of histones (along with other proteins) and repress target genes. These histone modifications cause chromatin condensing, which results in or maintains gene silencing of genes encoding proteins involved in maintaining cell identity. PcG proteins dynamically interact with their target genes, enabling precisely controlled differentiation processes [32, 33]. Undefined mechanisms govern the displacement of PcG proteins and recruitment of various subsets of target genes during cell fate decisions. Long non-coding RNAs and transcription factors are also emerging as potential regulators of cell fate decisions [33].

PRCs are primary chromatin modifiers that repress gene expression by affecting the global epigenetic landscape. There are two main PcG complexes, and each one consists of several different subunits. The histone code is established by PRC2 and deciphered by PRC1. The mammalian PRC2 complex is composed of Enhancer of Zeste Homologue 2 (EZH2), Embryonic Ectoderm Development (EED), Suppressor of Zeste Homologue 12 (SUZ12), and Retinoblastomabinding Protein 4 (RBBP4) or RBBP7 [34].

The hallmark of inactive chromatin is trimethylation of lysine 27 of histone 3 (H3K27me3), which is carried out by EZH2. EED and SUZ12 lack histone methyltransferase activity; nevertheless, these subunits are necessary for EZH2's histone methyltransferase action. PRC1 recognizes the restrictive histone marks generated by PRC2 and stabilizes the inactive state to preserve transcriptional memory [30].

TrxG and PcG genes are commonly reported to be altered or disregulated in cancer, supporting the idea that cancer is a disease of stem cells and differentiation [32].

2.6 Targeting EZH2 in Cancer

The enhancer of zeste homolog 2 (EZH2) is one of the four core members and the catalytic subunit of PRC2, which functions as a transcriptional repressor. Through its C-terminal SET domain, EZH2 catalyzes H3K27me3. This epigenetic alteration that compacts chromatin compaction and renders it hardly accessible to the transcriptional machinery consequently results in the silencing of target genes [35] (Figure 3).

In a context-dependent manner, EZH has been demonstrated to modify the tumor metastatic landscape, regulate the tumor microenvironment, and affect cell fate decisions. EZH2 has been identified as an oncogenic factor in several types of cancer [36–38]. Its overexpression promotes cancer development and progression by altering chromatin through epigenetic activation of carcinogenic signaling pathways and the silencing of tumor suppressor genes [39, 40]. EZH2 has a central function to repress genes involved in differentiation, favoring stemness retention [40]. Converging lines of studies have emphasized that EZH2 directs cell plasticity, fosters intertumoral heterogeneity, promotes cancer progression by inducing invasion and metastasis, and consequently leads to a worse prognosis [41, 42]. Overexpression of EZH2 has been related to invasion and metastasis in bladder cancer. Previous research has demonstrated that EZH2 overexpression has function in the metastatic cascade of bladder carcinomas, and selective depletion of EZH2 activity significantly hinders the migration of cancer cells and the metastasis of tumors [43–45]. The research has emphasized the function of EZH2 inhibition in tumor suppression in MIBC, supporting the less

aggressive phenotype through a basal-to-luminal shift in the transcriptional program and a clear enrichment of an immune-activating transcriptional signature [46].

Cancers harboring gain-of-function mutations in EZH2 and loss-of-function mutations in the SWI/SNF complex and KDM6A are considered responsive to EZH2-mediated epigenetic therapy by stimulating an immunological response that promotes tumor cell differentiation and death [47]. It has been shown that EZH2 knockdown reduces tumor progression in various malignancies [48–50]. Furthermore, the selective apoptosis-inducing activity of EZH2 inhibition on cancer cells rather than in normal cells portrays EZH2 as a promising therapeutic target [51].

Considering the potential role of EZH2 as a cancer driver, therapeutic approaches have been developed targeting EZH2 using specific pharmacologic inhibitors. S-adenosylmethionine (SAM), a component of the methionine cycle, provides the methyl group that EZH2 utilizes to modify the target genes. A crucial step in reducing EZH2 enzyme activity is to prevent the formation and utilization of methyl groups [52]. Consequently, diverse SAM-competitive EZH2 inhibitors have been designed. Among these, the FDA has authorized tazemetostat due to its promising results that have been acquired in human phase 1 and 2 trials [53, 54]. GSK-126, another highly selective SAMcompetitive inhibitor of EZH2 methyltransferase activity, has been demonstrated to cause loss of global H3K27 methylation and reactivation of PRC2-repressed genes [55]. Although the phase I clinical trial of GSK-126 was less encouraging (NCT02082977), the subsequent investigation reveals a potential mechanism underlying the unsatisfactory results and provides a usable combinational approach to tackle it [56].

In general, the response to EZH2 inhibition in solid tumors is not as robust as the hematological ones. In this context, combining epigenetic drugs with other treatments represents a rational approach for elevating the sensitivity of cancer cells to therapy and overcoming acquired resistance mechanisms. Recently, increasing evidence suggested that employing EZH2 inhibitors in combination with other treatments provided more potent antitumor effects in solid tumors [16].

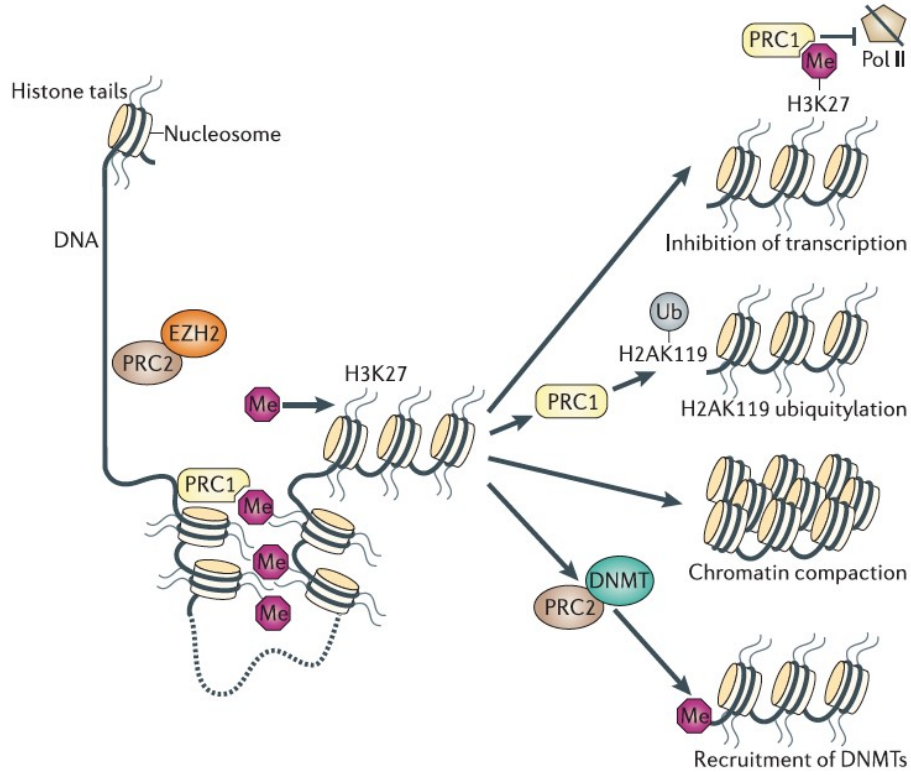


Figure 3. Gene silencing via Polycomb protein complexes

EZH2-mediated methylation of histone proteins is induced upon binding of the PRC2 initiating complex to the PcG target genes, particularly at H3K27. PRC1 can identify the H3K27me3 mark via the Polycomb chromodomain. This connection might enable extensive methylation across large chromosomal regions by bringing nearby nucleosomes into proximity to the PRC2 complex. Although the specific mechanisms underlying PRC-mediated gene silencing remain unknown, they are assumed to involve direct inhibition of the transcriptional machinery, ubiquitylation of H2AK119 by PRC1, chromatin condensing, and PRC2's recruitment of DNA methyltransferases to target gene loci (Adapted from [34], permission to use: license number: 5631831132168).

2.7 Retinoic Acid Signaling

Retinoic acid (RA) signaling represents one of the most crucial biological pathways, mediated by the interaction of RA with nuclear receptors to regulate gene transcription. RA signaling molecules constitute genetic communication networks regulating crucial cellular functions in embryonic development, organogenesis, organ homeostasis, metabolic regulation, cell proliferation, differentiation, and death [57]. The interest in RA in cancer has risen over the last decade

as an outcome of numerous findings regarding the biological function of RA in influencing the biology of tumor-initiating cells [58, 59], and cancer resistance [60], as well as cell reprogramming and differentiation [61, 62]. Utilization of RA in clinical settings has demonstrated major pharmacological restrictions, including the factors that obstructed its administration, reducing its therapeutic efficacy, and the side effects driven by its systemic delivery. All these challenges encouraged researchers to formulate novel and more tolerated synthetic retinoid molecules [63].

The retinoids family consists of three units, including a hydrophobic end group, a core polyene chain, and a polar region (typically a carboxyl group) [64]. All-trans retinoic acid (ATRA), 9-cis retinoic acid, and 13-cis retinoic acid represent distinct forms of natural retinoids. Various synthetic retinoids have been developed to minimize the systemic toxicity of the natural retinoids and improve their stability and selectivity against certain RAR subtypes [63]. Since RA affects several signaling pathways, it has been employed for many years as a part of combination treatments.

Retinoids, which are retinoic acid derivatives, have been widely recognized as tumorsuppressive and chemopreventive agents owing to their central roles in apoptosis and differentiation [65, 66]. In addition, current research has revealed the important role of RA signaling in the specification, homeostasis, and regeneration of urothelium [67–69].

Retinoid-mediated gene transcription is orchestrated by chromatin remodeling and modifying complexes that change the chromatin environment [70]. As transcription factors, retinoid X receptor (RAR/RXR) and retinoic acid receptor (RAR/RXR) complexes control the transcriptional activation of RA target genes by attracting specific co-regulators to the RA response elements [71]. Unliganded RAR-RXR dimers form complexes with co-repressors, including histone deacetylases, which efficiently lead to condensation of the chromatin and consequently repression of target genes. Ligand binding elicits a conformational shift that releases co-repressors and recruits co-activators, such as histone acetyl transferases (HAT), to decompress chromatin by reducing PRC2 binding at these regions and ultimately activating transcription of target genes [72, 73] (Figure 4). In

the status of derepression of the target genes, PRC2 binding is reduced at these regions, highlighting the co-repressor function of PRC2 in RA-mediated gene expression.

Fenretinide (N-4-hydroxyphenyl-retinamide) is a synthetic form of ATRA that has exhibited substantial cytotoxic potency on cancer cells in *in vitro* studies and potential tumorsuppressive properties with a favorable toxicological profile in preclinical models [74–76]. Retinoids' pleiotropic actions and capacity to induce cell differentiation and death hold promise for cancer treatment and chemoprevention.

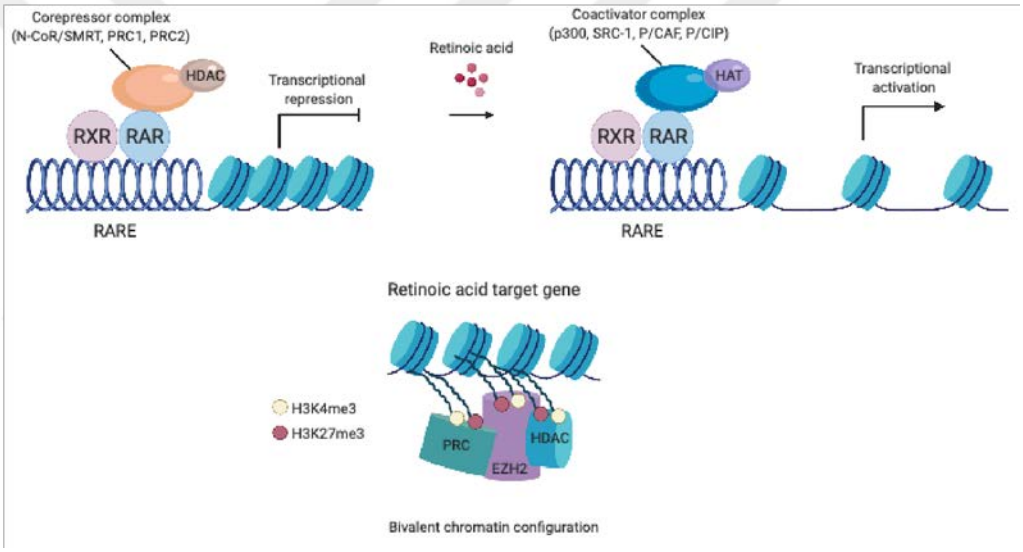


Figure 4. Molecular mechanisms of retinoic acid signaling

RARs are capable of acting as molecular switches. Unliganded RARs complex with co-repressors, rendering DNA inaccessible for transcription. Upon RA binding, transcription is activated by the release of corepressors and the attraction of co-activators. Several RA target genes reside within bivalent chromatin domains. The activation or repression of gene transcription is mediated by relative alterations in the various histone modifications [77].

3 MATERIALS AND METHODS

This chapter outlines the materials and various procedures employed to perform the experiments and analyze the acquired data.

3.1 Research Type

The research is an experimental study.

3.2 The Place and Time of the Research

This study was conducted at the Izmir Biomedicine and Genome Center (iBG) between 2018 and 2023.

3.3 Research Material

Human bladder cancer cell lines 5637 and HT1376 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Human bladder cancer cell lines J82 and T24 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States).

3.4 Variables of the Research

While drug treatments and duration of treatment are independent variables, changes in cell viability, apoptosis, epigenome, and transcriptome status constitute the dependent variables in this study.

3.5 Data Collection Tools

3.5.1 Materials

3.5.1.1 Laboratory Equipment and Consumable Materials

Laboratory equipment and consumable materials used in this study are shown in Table 1 and Table 2, respectively.

Table 1. Laboratory equipment

Equipment	Company
2100 Bioanalyzer	Agilent Technologies

7500 Fast Real-Time PCR System	Applied Biosystems
Benchtop orbital shaker	Thermo Scientific MaxQ 4000
Bioruptor Pico Sonicator	Diagenode
Centrifuges	Eppendorf 5810 R
Cool rack	Corning
Covaris sonicator	S220 High Performance Ultrasonicator
Digital heating and shaking drybath	Thermo Fisher Scientific
DynaMag 2 magnet	Thermo Fisher Scientific
Electronic pipettor	Gilson
Flow cytometer	BD LSR Fortessa
Freezer (-20)	Bosch, GSN 51 AW
Freezer (-80)	New Brunswick
Fume hood	Kötterman
Gel documentation system	BIO-RAD Gel Doc XR+
Hemocytometer	ISOLAB
Human cell culture incubator	Memmert
Light microscope	ZEISS Vert.A1
Magnetic stirrer	Thermo Barnstead (BS-SP131320-33Q)
Micro-centrifuge	Thermo Scientific Micro CL 17R
Micropipettes	Gilson
Microplate spectrophotometer	Infinite 200 Pro Microplate Reader, TECAN
Mini Electrophoresis System	Thermo Scientific OWL B1A
Mini vortex mixer	Thermo Scientific LPMixer
Mr. Frosty freezing container	Thermo Scientific

Nanodrop 2000	Thermo Scientific
Equipment	**Company**
Nitrogen tank	CHART
pH meter	Hanna ELED
Power Pac Basic Power Supply	BioRad
Safe 2020 Class II Biological Safety Cabinets	Thermo Scientific
SPS High-Performance D300 Digital Dispenser	HP, Inc., Specialty Printing Systems, 1070 NE Circle Blvd Corvallis., OR 97330
Thermal cycler	Simpleamp A24811
Tube rotator	Thermo Scientific Eled 88881002
Vacuum filtration system	Milipore
Water tank	Nuve NB9
Western Blot Membrane Imaging System	LI-COR Odyssey CLX
Western blot system	BIO-RAD

Table 2. Consumable materials

Materials	**Company**
Cell culture dishes	Corning
Cell scrapers	ISOLAB
Cryogenic tubes	Corning
Disposable pipette tips	Neptune
DNA LoBind Tube	Eppendorf
Eppendorf tubes	Thermo Fisher Scientific

Falcons	SPL
Greiner 384 flat bottom black polystyrol	Sigma-Aldrich
Materials	**Company**
MicroAmp Fast Optical 96-Well Reaction Plate	Thermo Fisher Scientific
Multiwell plates	Corning
Nitrocellulose membrane	BIO-RAD
PCR tubes	Neptune
Qubit assay tubes	Invitrogen, Q32856
Serological pipettes	SPL

3.5.1.2 Commercial Kits

Commercial kits used in this study are shown in Table 3.

Table 3. Commercial kits

Kit	**Company**
ChIP DNA Clean and Concentrator Kit	D5205, Zymo Research
Dead Cell Apoptosis Kit with FITC Annexin V and PI for Flow Cytometry	V13242, Invitrogen
DNA Clean & Concentrator Kit	D4034, Zymo Research
EZ-PCR Mycoplasma Detection Kit	20-700-20, Biological Industries
FastStart Essential DNA Green Master	06402712001, Roche
Maxima First Strand cDNA Synthesis Kit	K1641, Thermo Fisher Scientific

Monarch Total RNA Miniprep Kit	T2010S, New England Biolabs
Pierce BCA Protein Assay Kit	23227, Thermo Fisher Scientific
Qubit dsDNA HS Assay Kit	Invitrogen
TruSeq Stranded Total RNA with Ribo-Zero H/M/R Sample Library Preparation Kit	Illumina

3.5.1.3 Chemicals

Chemicals used in this study are shown in Table 4.

Table 4. Chemicals

Chemical	**Company**
10% APS	1610, Biofroxx
40% Acrylamide/Bis Solution 37.5:1	1610148, BIO-RAD
Acetic Acid	27225, Sigma-Aldrich
Acetone	179124, Sigma-Aldrich
Agarose	A9539, Sigma-Aldrich
Blotting-Grade Blocker	170-6404, BIO-RAD,
B-mercaptoethanol	444203, Sigma-Aldrich
Bovine Serum Albumin (BSA)	A9418, Sigma-Aldrich
Bromophenol blue	B0126, Sigma-Aldrich
Color Protein Standard	P7719S, BioLabs
Dimethyl sulfoxide (DMSO)	276855, Sigma Aldrich
DTT	1114GR005, Neofroxx
Dynabeads Sheep Anti-Rabbit IgG	11204D, Invitrogen
EDTA	SRE0033, Sigma-Aldrich
EGTA	324626, Sigma-Aldrich

Ethanol	100983.2511, Merck
Fenretinide	390900, Merck
Formaldehyde	252549, Sigma-Aldrich
Glycerol	G9012, Sigma-Aldrich
Glycine	G7403, Sigma-Aldrich
GSK-126	S7061, Selleckchem
HCl	32033, Sigma-Aldrich
Isopropanol	563935, Sigma-Aldrich
LiCl	L9650, Sigma-Aldrich
Chemical	**Company**
Methanol	32213, Sigma-Aldrich
Na deoxycholate	D6750, Sigma-Aldrich
NaCl	S5886, Sigma-Aldrich
$NaHCO_3$	S5761, Sigma-Aldrich
PAO	P3075, Sigma-Aldrich
Phosphate Buffer Saline (10X)	70011036, Thermo Scientific
Ponceau	P3504, Sigma-Aldrich
Propidium Iodide	P3566, Invitrogen
Protease Inhibitor Cocktail	11697498001, Roche
Proteinase K	T2002-1, New England BioLabs
Resazurin	R7017, Sigma-Aldrich
RNase A	10109169001, Roche
SDS	24730020, Invitrogen
Stacking Gel Buffer	1610799, BIO-RAD

Sulfuric acid	339741, Sigma-Aldrich
TCA (Trichloroacetic acid)	T6399, Sigma-Aldrich
TEMED	T22500, Sigma-Aldrich
Tris	648314, Sigma-Aldrich
Tris Acetate-EDTA (TAE) Buffer (10X)	T9650, Sigma-Aldrich
TritonX-100	T8787, Sigma-Aldrich
tRNA	R8508, Sigma-Aldrich
Tween-20	P1379, Sigma-Aldrich

3.5.1.4 Cell Culture Reagents

Cell culture reagents used in this study are shown in Table 5.

Table 5. Cell culture reagents

Reagent	**Company**
Dulbecco's Modified Eagle Medium (DMEM)	41965039, Gibco
Fetal Bovine Serum (FBS)	10500064, Thermo Scientific
Freezing Medium	12648010, Thermo Scientific
Gibco Trypan Blue	15-250-061, Thermo Scientific
Penicillin/Streptomycin	15140122, Thermo Scientific
Phosphate-Buffered Saline (PBS)	70011036, Thermo Scientific
Roswell Park Memorial Institute (RPMI) Medium	21875, Thermo Scientific

TrypLE Express Enzyme	12604013, Thermo Scientific

3.5.1.5 Buffers

Buffers used in this study are shown in Table 6.

Table 6. Buffers

Component	**Final Concentration**
ChIP Buffers	
Elution buffer	1 M $NaHCO_3$, 1% SDS
High salt buffer	0.5% DOC, 0.5% NP-40, 0.25 M LiCl, 1 mM EDTA, 10 mM Tris pH 8.0
Paro Rinse 1	10 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 0.5 mM EGTA, and 0.25% Triton X-100
Paro Rinse 2	10 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM EGTA, and 200 mM NaCl
Component	**Final Concentration**
SDS lysis buffer	0.1% SDS, 0.1% DOC, 1% Triton X, 1 mM EDTA, 500 mM NaCl, and 50 mM HEPES/KOH
Western Blot Buffers	
Hypotonic lysis buffer	10 mM Tris-Cl pH 8.0, 1 mM KCl, 1.5 mM MgCl2
Laemmli sample buffer (4X)	62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.05% Bromophenol Blue, 50 mM DDT
RIPA lysis buffer	0.15 M NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris (pH 7.5), 2 mM EDTA
Running buffer (10x)	0.25 M Tris HCl, 1.92 M Glycine and 1% (w/v) Sodium Dodecyl Sulfate (SDS); pH 8.3
Transfer buffer (10x)	250 mM Tris, 1.92 M Glycine and 20% methanol (v/v); pH 8.3

3.5.1.6 Antibodies

Antibodies used in this study are shown in Table 7.

Table 7. Antibodies

Antibody	Company
Anti-mouse IgG (H+L) (DyLight™ 680 Conjugate)	5470, Cell Signaling Technology
Anti-rabbit IgG (H+L) (DyLight™ 800 4X PEG Conjugate)	5151, Cell Signaling Technology
EZH2	5246, Cell Signaling Technology
H3K27me3	9733, Cell Signaling Technology
Histone H3	39763, Active Motif
β-Actin Mouse	3700, Cell Signaling Technology
β-Actin Rabbit	4970, Cell Signaling Technology

3.5.1.7 Primers

The sequences of RT-qPCR and H3K27me3 ChIP-qPCR primers are provided in Table 8 and Table 9, respectively.

Table 8. RT-qPCR primers

Gene	Forward Primer Sequence (5'->3')	Reverse Primer Sequence (5'->3')
AKR1C2	GGGTTCCACCATATTGATTCT	CACTGCCATCTGCAATCT
ATF3	TTCTGAGCCCGGACAATACAC	AAGAACGAGAAGCAGCATTTGAT
CASP4	TTCCCTATGGCAGAAGGCAAC	CCACCAAGTTATCCAAAACACCA
CDH1	AGTGACTGATGCTGATGCCC	CCAAGCCCTTTGCTGTTTTCA
CDH2	CAGAGTTTACTGCCATGACGTTTT	ACCGTCCAGTAGGATCTCCG
CDKN1C	GCGGCGATCAAGAAGCTGT	ATCGCCCGACGACTTCTCA

FOXA1	CTACTACGCAGACACGCAGG	TCATGTTGCCGCTCGTAGTC
GADD45A	AGAGCAGAAGACCGAAAGGATG	TTCGTACACCCCGACAGTGA
GAPDH	AAGGTCGGAGTCAACGGATTT	TGAAGGGGTCATTGATGGCA
GATA4	CGACACCCCAATCTCGATATGTT	ACAGATAGTGACCCGTCCCA
HSPA1A	AGCTGGAGCAGGTGTGTAAC	CAGCAATCTTGGAAAGGCCC
HSPB8	CTCCTGCCACTACCCAAGC	GGCCAAGAGGCTGTCAAGT
ITGB1	ATGAATGAAATGAGGAGGATTACTTCG	AAAACACCAGCAGCCGTGTAAC
KRT18	AACAACACCTGCTGTCCGTG	CCGGTAGTTGGTGGAGAAGG
PTGS2	CCCTTGGGTGTCAAAGGTAAAA	AACTGATGCGTGAAGTGCTG
STAT3	ATCAAGCAGTTTCTTCAGAGCA	AAGGCGTGATTCTTCCCACA
TGFBR2	CTGGGGGCTCGGTCTATGA	CCTCCATTTCCACATCCGAC
UPK1A	GGGATCTCCAGTTGTGGTGG	GCCTGAGACTCCCATCAGTG
VIM	GGCGAGGAGAGCAGGATTTC	AGTGGGTATCAACCAGAGGGA

Table 9. H3K27me3 ChIP qPCR primers

Gene	Forward Primer Sequence (5'->3')	Reverse Primer Sequence (5'->3')
AKR1C2	ATCAATATGGTGGAACCCGGC	TGCTTGTGCCCGAACTAACT
ATF3	GGTCTCAGCTCGAATCTCGG	AATGGGGACGCAATGACCAC
GLIS2	AAAAACAGATGTGTTACCCCTCA	TCTCCCCTAGAGCCTTCCAG
HSPB8	AAAATGTTAGAGGGTGCGGGG	TGCCAATTGCGCTATCCTGT
IGFBP3	GATGGGGCGACAGTACACG	CCGCCCAGATGCGAGC
MEIS3	GGCACCTTCCTCTCATCAGG	CGTTCTAGCCTCTTCCCGTG
NDRG1	GTGTGTGCTACGGGACTGAA	GGTTGGTAGAGGTGAGTGGC

PTGS2	CCGTGTCTGGTCTGTACGTC	ACAGCCTATTAAGCGTCGTCA
SERPINE2	GGACCCAAGGACCCAACTTT	TTTCTTCCTCGCTGTTGGGG

3.5.2 Methods

3.5.2.1 Cell Culture

Human bladder cancer cell lines 5637 and HT1376 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Human bladder cancer cell lines J82 and T24 were purchased from the American Type Culture Collection (ATCC, VA, United States). HT1376, J82, and T24 cells were cultured in DMEM, whereas 5637 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) with streptomycin (100 μg/ml) and penicillin (100 units/ml) under standard culture conditions (5% CO_2, 37 °C). The cells were treated with TrypLE Express Enzyme to perform serial passages. All cell lines were subjected to routine mycoplasma testing.

3.5.2.2 Reagents

Fenretinide and GSK-126 were dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stocks and diluted directly in the culture medium for further experiments.

3.5.2.3 Cell viability assays

The cells were seeded on the 384-well plates at 50 μL per well at concentrations of serial dilution. The optimal cell seeding density was determined by cell number titration. The cells were treated with serial dilutions of drugs. After 24 h of incubation, 10 μL of 0.05 mg/mL resazurin was added to each well, and the plates were further incubated for 4 h at 37 °C. Fluorescence was read at an excitation wavelength of 570 nm and an emission wavelength of 600 nm on an Infinite 200 Pro microplate reader. For viability assays, cells were seeded at an optimal density and treated with DMSO, fenretinide, or GSK-126 for the indicated periods. To calculate the yield percent survival, the results were normalized to the vehicle control. The concentrations were converted to a log scale

for plotting as log (inhibitor) vs. response-variable slope. Analyses were performed using the GraphPad Prism software.

3.5.2.4 Drug combination analysis

Cells were seeded on 384-well plates in 50 µL of culture medium in triplicates according to optimal seeding density calculations. After allowing them to attach for 24 h, the cells were treated with the indicated serial dilution concentrations of fenretinide and GSK-126, alone or in combination. DMSO and PAO were used for vehicle control and positive control, respectively. Drug treatments were performed using the SPS High Performance D300 Digital Dispenser. After 72 h of incubation, 10 µL of 0.05 mg/mL resazurin was added to each well and incubated at 37 °C for 4 h. The plates were read using the Infinite 200 Pro microplate reader. The Bliss synergy score was calculated using the SynergyFinder v2.0 web-based tool with default parameters.

3.5.2.5 Apoptosis assay

Cells were seeded into 60-mm dishes at the optimal density. After an overnight incubation, the cells were treated with drugs. The apoptosis assay was performed with the Dead Cell Apoptosis Kit with FITC Annexin V and PI. Briefly, after the incubation period, the cells were harvested, washed in cold phosphate-buffered saline (PBS), and stained with 5 µL of Annexin FITC and 1 µL of PI (100 µg/mL) in 100 µL of 1X annexin-binding buffer. After 15 minutes of incubation, the cells were analyzed by flow cytometry, measuring the fluorescence emission at 530 nm and >575 nm. The data were analyzed with the FlowJo software.

3.5.2.6 Cell cycle analysis

Cultured cells were collected and washed in PBS. The cells were fixed in pre-cooled 70% ethanol by adding dropwise to each sample and then incubated for at least 30 min at 4 °C. After being washed with PBS, the cells were stained with RNase A (100 µg/mL), propidium iodide (50 µg/mL), and 0.1% Triton X-100. The data was acquired on the flow cytometer and analyzed by FlowJo software.

3.5.2.7 Scratch assay

To create a confluent monolayer, the cells were seeded into 6-well plates and incubated overnight. The scratch has been created with a 200-µL pipette tip. After washing twice with PBS to eliminate debris, the cells were cultured with low serum-containing media for varying time points. Different reference points were selected from a single well. The percentage of wound healing area was analyzed by Image J software.

3.5.2.8 RNA isolation, cDNA synthesis, and RT-qPCR

RNA was extracted using the Monarch Total RNA Miniprep Kit according to the manufacturer's instructions. The RNA concentration was quantified with a NanoDrop 2000 spectrophotometer. The Maxima First Strand cDNA Synthesis Kit was used to reverse-transcribe RNA to cDNA. Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) was performed with the FastStart Essential DNA Green Master Kit on a 7500 fast real-time PCR system. Relative gene expressions were calculated by the $2^{-\Delta\Delta CT}$ method [78] normalizing to the housekeeping gene GAPDH using three technical replicates. All primers were designed by NCBIPrimer Blast or adapted from the literature and synthesized by Oligomer.

3.5.2.9 RNA-seq and RNA-seq data analysis

Total RNA was extracted using the Monarch Total RNA Miniprep Kit according to the manufacturer's recommendations. Total RNA integrity was checked using a 2100 Bioanalyzer. Paired-end libraries were prepared using the TruSeq Stranded Total RNA with Ribo-Zero H/M/R Sample Library Preparation Kit and sequenced on the Illumina Novaseq 6000.

The FASTQC tool (v0.11.9; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check the quality of raw sequencing data. Adapter removal and quality trimming steps of sequencing reads were performed with Trimmomatic (v0.36) [79]. The human reference genome (GRCh38) in FASTA format and related gene annotations in General Transfer Format were manually downloaded from the GENCODE (Release 32) project web site

(https://www.gencodegenes.org). The sequenced reads were aligned to the GRCh38 with Rusbread v2.0.3 package [80] of the R v4.1.2 statistical computing environment (https://www.r-project.org/). SAM Tools v1.3.1 were utilized to sort and index the BAM files [81]. The gene expression levels were calculated with the featureCounts function of Rsubread package [82]. The "fpkm" function of the edgeR package (v3.24.3) was used to calculate the fpkm values of each gene across samples [83]. The genes meeting the criteria of log2 fold change (log2 FC)> 0.5 between groups were considered differentially expressed. Graphical representations were generated using the R statistical computational environment. The R-package clusterProfiler (version 3.18.0) was used to identify and visualize the enrichment of Gene Ontology (GO) terms in the sets of genes of interest [84]. Graphics associated with RNA-seq data were generated with the R package ggplot2 (https://ggplot2.tidyverse.org/).

3.5.2.10 Western blot analysis

Cellular proteins were extracted using RIPA lysis buffer supplemented with protease inhibitors and cleared by centrifugation. Nuclear proteins were extracted by the histone acid extraction method [85]. Briefly, the cell pellet was lysed (30 min at 4 °C) in a hypotonic lysis buffer supplemented with DTT (1 mM) and a complete protease inhibitor cocktail (PIC). Following centrifugation, the intact nuclei were resuspended in H_2SO_4 (0.2 M). Samples were incubated overnight, and trichloroacetic acid (TCA) precipitation was performed to collect histones. Samples were incubated on ice (30 min), and the histone pellet was washed with ice-cold acetone to remove the acidic fraction from the solution. Samples were air dried, and the histone pellet was dissolved in ddH_2O. The BCA Protein Assay Kit was used to determine the protein concentrations.

Equal amounts of protein extracts were denatured with heating (95°C for 5 min) in reducing agents (Laemmli buffer). Samples were separated on the acrylamide gel and transferred to nitrocellulose membranes. Blotting was confirmed by staining the membranes with a Ponceau S solution. The membranes were blocked in TBST with 5% milk for 1 hour. The membranes were incubated overnight at 4 °C with the following primary antibodies: EZH2, H3K27me3, β-Actin, and Histone H3. After washing with TBST, the corresponding secondary antibodies were incubated

on the membranes for 1 hour at room temperature. Protein bands were visualized with the Odyssey CLX imaging system. β-Actin or histone H3 were used as loading controls on blots.

3.5.2.11 Chromatin Immunoprecipitation (ChIP)

For each ChIP, approximately 20 million cells were crosslinked with 1% formaldehyde (10 min) and quenched by glycine (125 mM). The cell pellet was lysed in Paro Rinse buffers and then in dodecyl sulfate (SDS) lysis buffer supplemented with PIC. The chromatin was disrupted by sonication using a Covaris sonicator (15 cycles of 140 W peak power, 15 duty factors, 200 cycles/burst for 60 sec) to obtain 200–500 bp-long fragments. Sheared chromatin was clarified by centrifugation (16,000 g, 10 min, at 4 °C). 10% of the sonicated lysate was stored for input control. After treating with RNase A (0.2 mg/ml) and Proteinase K (200 μg/ml), cross-linking was reversed by adding SDS (1%) and NaCl (100 mM) and heating at 55 °C (2 h) and 65 °C (overnight). The shearing quality was confirmed on the agarose gel. The input DNA was extracted using the Zymo DNA Clean and Concentrator Kit. The magnetic beads were blocked with BSA (1 mg/ml) and tRNA (1 mg/ml) solutions. Pre-clearing was performed with pre-blocked magnetic beads. The chromatin sample was incubated with antibody overnight (4°C, with overhead shaking), then incubated with pre-blocked magnetic beads (3 h at 4°C). After washing with low and high salt buffers, the immunoprecipitated DNA was eluted off the magnetic beads with elution buffer and reverse cross-linked by heating at 55°C for 2 h and 65 °C for 16-18 h. The immunoprecipitated DNA was extracted using the ChIP DNA Clean and Concentrator Kit. H3K27me3 content at the promoters of specified genes was calculated by RT-qPCR with primers designed using the ChIPseq data available at Cistrome Data Browser [86]. The enrichment of immunoprecipitated DNA was calculated using the following method: The adjusted Ct value for the input sample was calculated as ($Ct_{10\%\ Input}$-log (10,2)). The ΔCt value of the input sample was calculated as ($Ct_{Adjusted\ Input}$ -$Ct_{ChIP\ Input}$). The percent input was calculated as (100∗2^(ΔCt)).

3.5.2.12 Chromatin immunoprecipitation sequencing (ChIP-Seq) analysis

A total of 50 ng of DNA was provided per ChIP sample to Macrogen for DNA library preparation using the TruSeq DNA sample preparation kit according to Illumina recommendations.

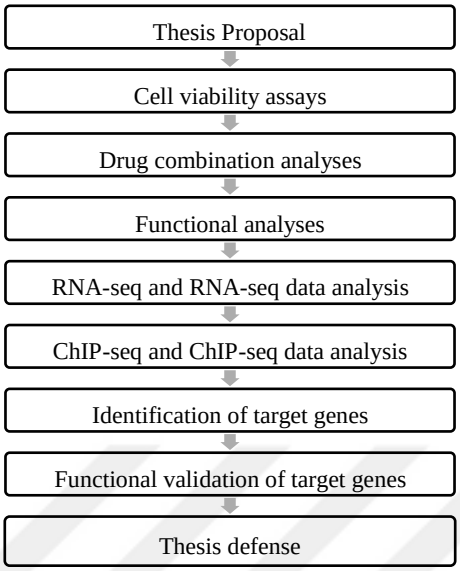
The ChIP DNA libraries were sequenced as paired-end 150-bp reads using the NovaSeq 6000 S4 sequencer (Illumina). Nextflow (version 22.10.5) nfcore/chipseq pipeline was used for ChIP-seq analysis (10.5281/zenodo.3240506). The Nfcore tool uses FastQC to read raw reads, then trims adapter by using Trim Galore, and uses Picard, SAMtools, BEDtools, and BAMtools to filter the data and prepare it for alignment. Broad peaks were called using MACS2 [87]. featureCounts was used to obtain count values of consensus peaks [82]. The parameters used to run nfcore/chip seq were as follows: genome=GRCh38, macs_gsize =2913022398, and *p*-value cutoff = 0.00001. A compute matrix was created with DeepTools' computematrix function to be used for plotHeatmap and plotProfile functions. After the bed files were obtained using the counts and annotation files, the compute matrix was obtained by using the bigWig files. After the compute matrix was obtained for the desired gene lists, chip-seq profiles and heatmaps for the samples were obtained. The Bioconductor Gviz package was used to visualize ChIP-seq signal data (https://bioconductor.org/packages/release/bioc/html/Gviz.html).

3.6 Evaluation of Data

With the exception of RNA-seq data, all data sets were analyzed using GraphPad Prism 7.0. Cell images were analyzed and quantified using ImageJ v1.45 software. All assays were performed in triplicate. All graphed data were presented as the mean ± standard deviation (SD). The statistical differences between groups were assessed by two-tailed Student's t-tests and Mann-Whitney U test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.7 Research Plan and Schedule

The plan and schedule of the research are represented in the following flow chart.



3.8 Limitations of the Research

Since our data regarding the alterations in the transcriptome and epigenome might not accurately reflect the real clinical scenario, further validation of findings regarding efficacy, tolerability, and pharmacokinetics in animal models is required to strengthen the significance of our findings.

3.9 Ethical Approval

This study was approved by the Non-Interventional Research Ethics Committee of Izmir Biomedicine and Genome Center (Number: 2023-007) on February 24, 2023.

4 RESULTS

4.1 Combination Treatment Inhibits Bladder Cancer Proliferation

Recent studies reveal that EZH2 has a crucial function in accelerating the progression of bladder cancer by encouraging invasion and metastasis, which eventually leads to a worse prognosis. To address the relevance of EZH2 to disease stage, we compared the EZH2 expression

levels in MIBC and NMIBC cases involving human primary tumors and cell lines. We observed that EZH2 expression is significantly increased in muscle-invasive tumors (Figure 5).

Using resazurin survival assays, we investigated the effects of fenretinide and GSK-126 on the proliferation of MIBC cells. 48 and 72 hours of treatment with varying concentrations of fenretinide or GSK-126 resulted in a dose-dependent decrease in MIBC cell proliferation (Figure 6 and Figure 7). We conducted our functional studies with 72 hours of incubation with these drugs since the overall responses to drugs required lower concentrations for 72 hours (Figure 6 and Figure 7).

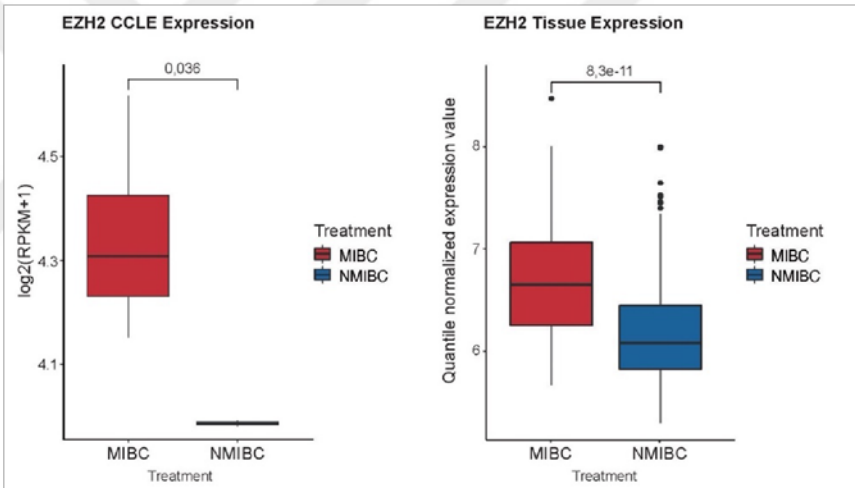
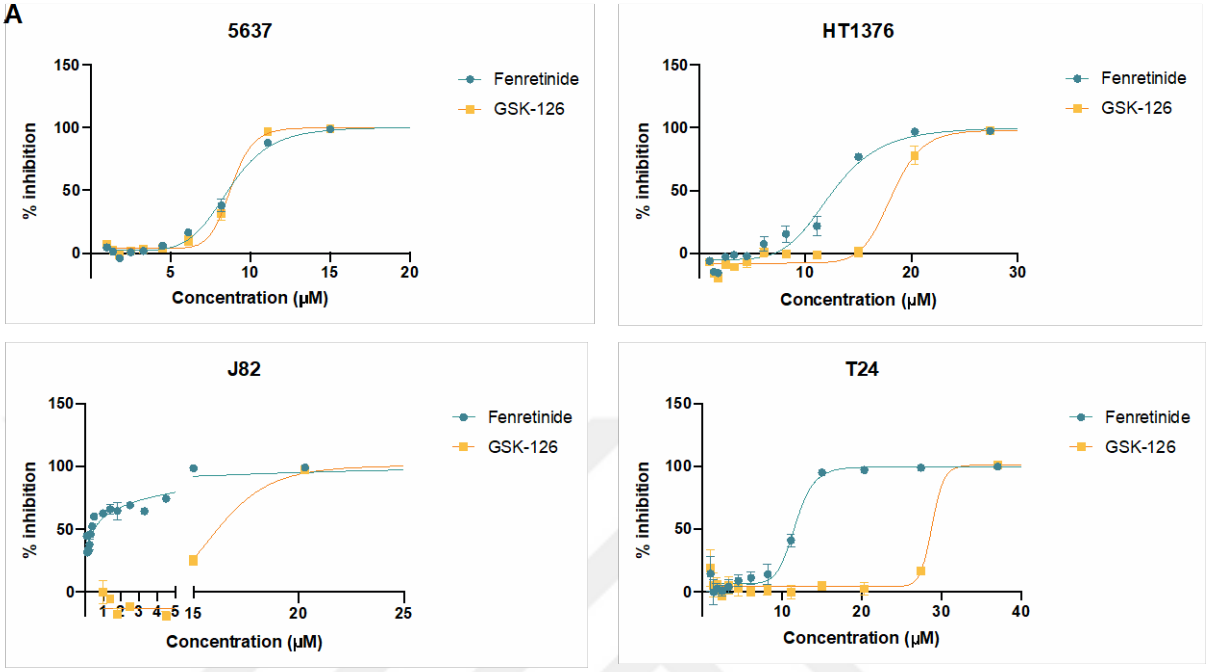


Figure 5. Association of EZH2 with bladder cancer progression

The left graph depicts the comparison of RNA expression levels of EZH2 in MIBC cell lines (5637, HT1376, J82, and T24) and NMIBC cell lines (RT4 and RT112) obtained from the publicly available Cancer Cell Line Encyclopedia (CCLE) database. The right graph represents the comparison of the EZH2 transcript levels derived from microarray profiling of NMIBC and MIBC tumor samples (GEO: GSE32894). P values of the t-test are displayed.

A

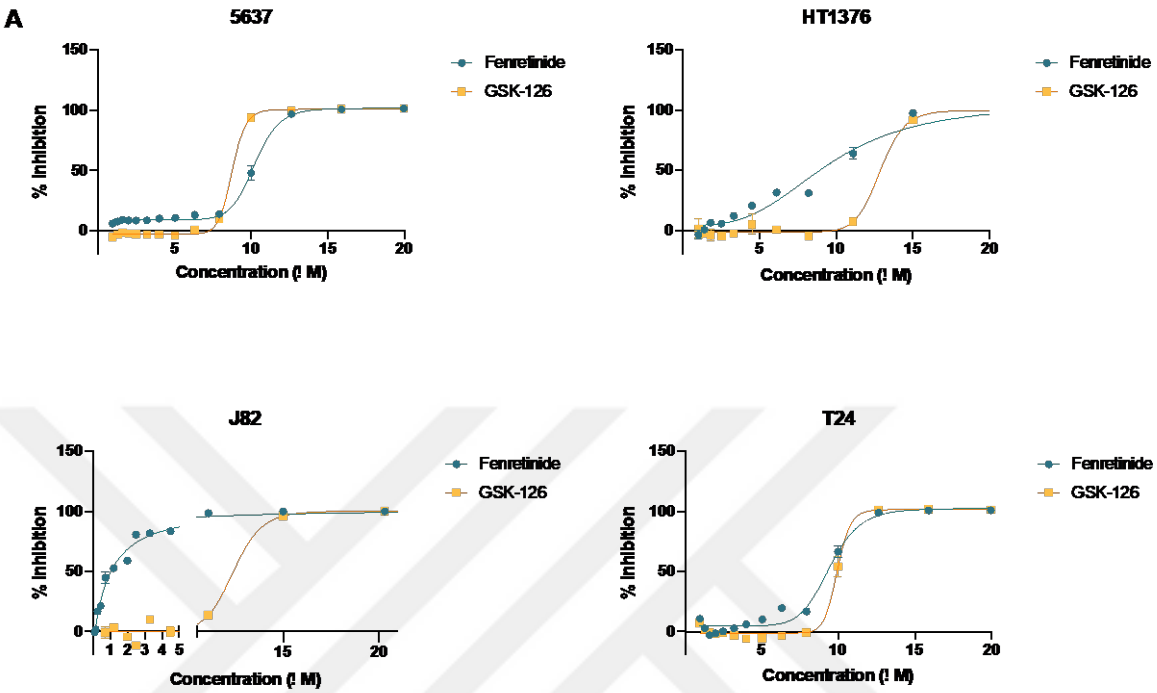


B

IC50 (μM)	5637	HT1376	J82	T24
Fenretinide	8.67	12.48	2.17	11.64
GSK-126	8.79	18.10	15.85	28.79

Figure 6. Inhibitory effects of 48 hours of drug treatment on MIBC cell viability

A) Dose-response curves of MIBC cells following treatment with the indicated concentrations of fenretinide or GSK-126 for 48 h. Data are presented as mean ± SD. B) IC50 concentrations of fenretinide or GSK-126 in MIBC cells following 48 h of treatment.



IC50 (μM)	5637	HT1376	J82	T24
Fenretinide	10.24	9.48	0.93	9.44
GSK-126	8.75	12.86	12.42	9.92

Figure 7. Inhibitory effects of 72 hours of drug treatment on MIBC cell viability

A) Dose-response curves of MIBC cells following treatment with the indicated concentrations of fenretinide or GSK-126 for 72 h. Data are presented as mean ± SD. B) IC50 concentrations of fenretinide or GSK-126 in MIBC cells after 72 h of treatment.

4.2 Fenretinide and GSK-126 Synergistically Inhibit Bladder Cancer Cell Viability

Considering that the PRC2 complex plays a co-repressor function in RA-mediated gene expression, we assume that inhibiting EZH2 activity could enhance retinoid action. To test this hypothesis, we subsequently conducted experiments to search for their possible interactions and investigate potential retinoid and EZH2 inhibition cooperation in bladder cancer. To assess the character of the interactions between fenretinide and GSK-126, we performed Bliss Independence model analyses, enabling drug interactions to be quantitatively assessed. The Bliss Independence

model, a frequently used concept in drug synergy research, is centered on the principle that the drugs function independently and do not interact with each other [88]. The SynergyFinder online tool was used to generate Bliss synergy plots. [89].

We tested the fenretinide and GSK-126 combination across a range of concentrations of each drug in MIBC cell lines. Based on a Bliss independence model combination matrix surface evaluation, the synergy scores of this drug combination were calculated as 38.09, 11.46, 4.67, and 57.01 for 5637, HT1376, J82, and T24 cells, respectively (Figure 8). A synergy score of >10 is typically considered to indicate a synergistic interaction, and this model clearly showed potent synergy for the drug combinations (except for the J82 cell line). These results indicate that the fenretinide and GSK-126 combination regime synergistically attenuates the viability of MIBC cells.

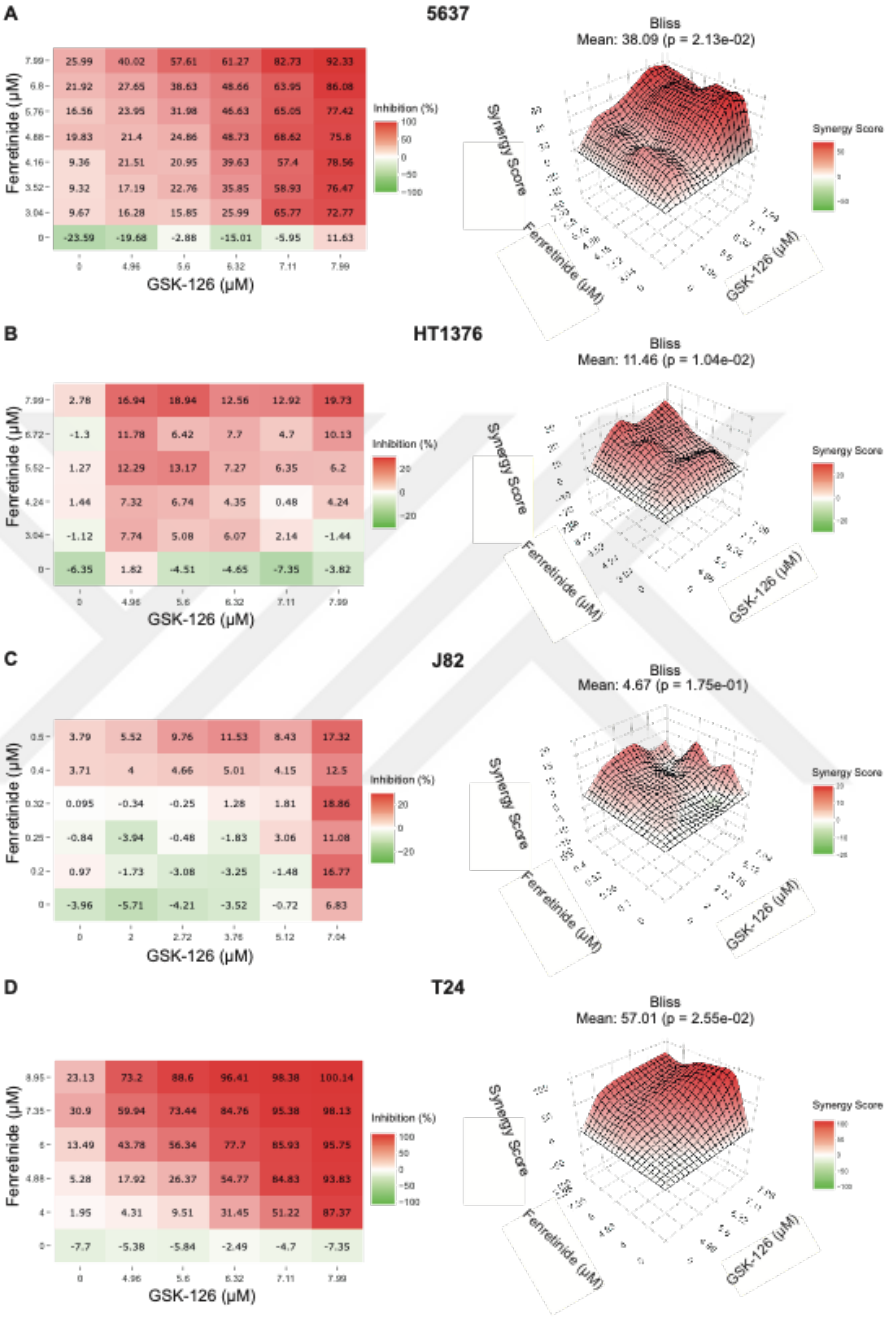


Figure 8. Synergistic inhibition of cell proliferation with fenretinide and GSK-126

Dose-response matrices and 3D Bliss synergy maps obtained by fenretinide and GSK-126 combination treatments (72 h) in MIBC cells. The color of the surface signifies the effect of the drug combination; while green indicates no effect, red indicates the maximum effect.

4.3 Combination Treatment Promotes the Anti-Oncogenic Signature

To delineate the alterations in tumor phenotypes with fenretinide and GSK-126 combination treatment, we conducted a series of functional analyses linked to apoptosis, cell cycle, and gene expression. For these analyses, we used 5 μM of each drug alone or in combination for 72 h, which corresponds to IC25–30 concentrations.

To assess the effects of drug combination on cell apoptosis, we stained the cells with Annexin V-FITC/propidium iodide (PI). The apoptosis analysis showed that the administration of drug combination significantly increased apoptotic cell frequency compared to single agents or control cells (Figure 9). Apoptotic bodies were also shown in fluorescence microscopy images (Figure 10).

To determine the changes in cell cycle, we next analyzed the cell cycle profiles of cells treated with the drug combination. Consistent with the apoptosis results, cell cycle analysis revealed that the combination treatment increased the frequency of T24 cells in the sub-G1 phase, slightly increased the cell population in the G0/G1 phases and decreased the cell population in the S phase. Collectively, these data suggest combination treatment induces cell cycle arrest more effectively compared with vehicle control, although it is not statistically significant (Figure 11). Thus, combined treatment significantly induced apoptosis and led to a moderate cell cycle arrest.

To investigate the effects of drugs on cell migration, we performed the scratch assay. We observed that the migration capacity of T24 cells decreased upon treatment in a time-dependent manner. Notably, combination treatment significantly suppressed the invasive capacity of cells compared with vehicle control and single treatments, especially 24 hours after the scratch (Figure 12).

To explore how the effects of single and combination treatments modulate gene expression levels, selected signature genes were analyzed by RT-qPCR. Supporting our findings from apoptosis assays, we observed that the expression levels of caspase activation-related and proapoptotic genes, CASP4 and GADD45A, are robustly augmented upon fenretinide-only and

combinatorial treatment as compared to control, suggesting the major function of fenretinide linked with apoptosis. Treatments with GSK-126 alone and in combination with fenretinide significantly increased the expression of CDKN1C, which has been linked to G1-phase cell cycle arrest (Figure 13). We evaluated the expression of a subset of marker genes reflecting epithelial or mesenchymal phenotypes and detected a significant increase in the expression level of the epithelial gene CDH1 by combination treatment. Among the genes associated with urothelial differentiation, the changes in the expression levels of FOXA1 and GATA4 were not uniform; however, expression of UPK1A was significantly increased by drug combination (Figure 13). Collectively, these findings emphasize that fenretinide and GSK-126 cooperate to modulate the expression of a group of genes linked to apoptosis, epithelial phonetype, and urothelial differentiation. Consequently, these data leverage evidence that simultaneous EZH2 inhibition and retinoic acid pathway activation might trigger an anti-oncogenic profile in MIBC.

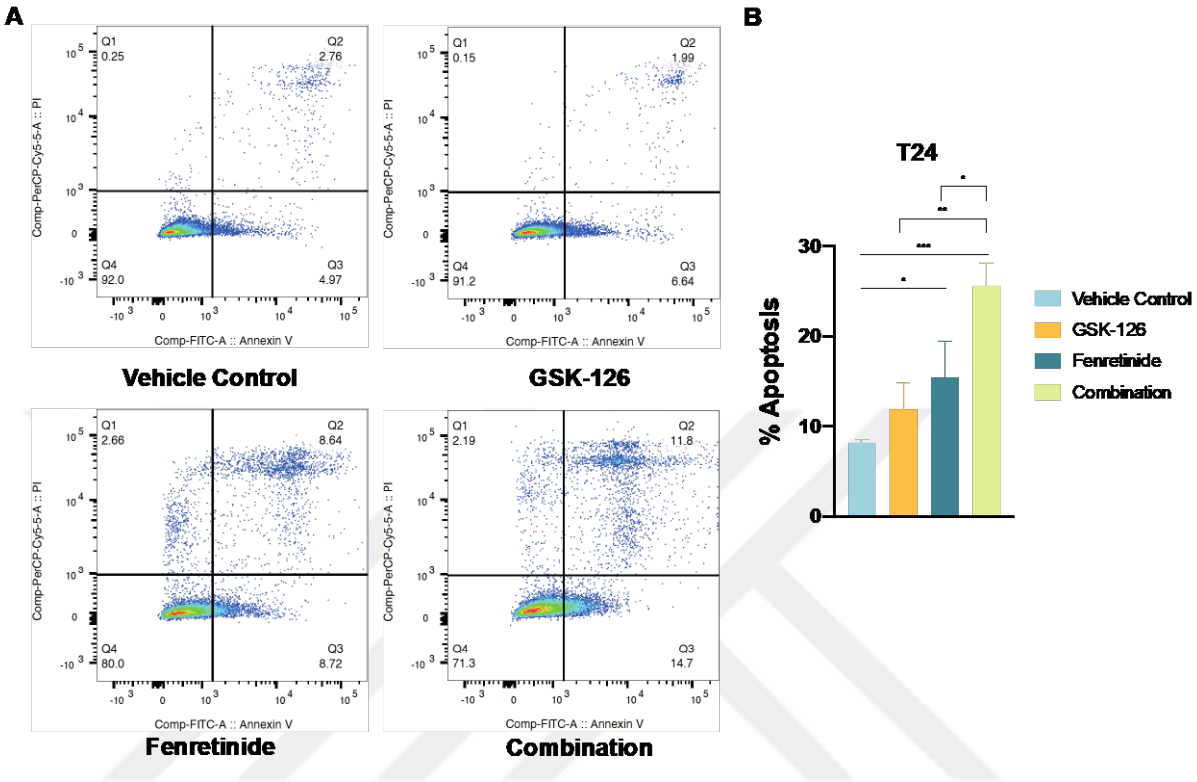


Figure 9. The effects of drug combination on cell apoptosis

A) Representative flow cytometric analysis of T24 cells treated with fenretinide (5 μM, 72 h) and GSK-126 (5 μM, 72 h) alone or in combination. (B) Quantification of apoptosis analysis performed with three biological replicates. Data are presented as mean ± SD (two-tailed unpaired t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

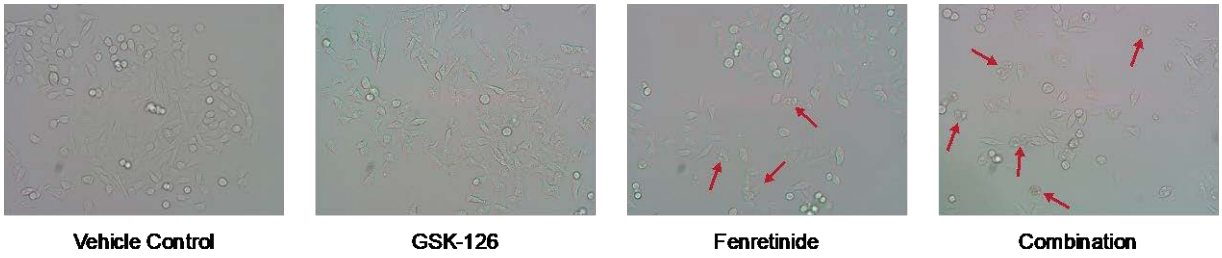


Figure 10. Apoptosis induced by combination treatment

Images representing the morphology of T24 cells treated with fenretinide (5 μM, 72 h) and GSK-126 (5 μM, 72 h) alone or in combination. Red arrows display blebs and apoptotic bodies.

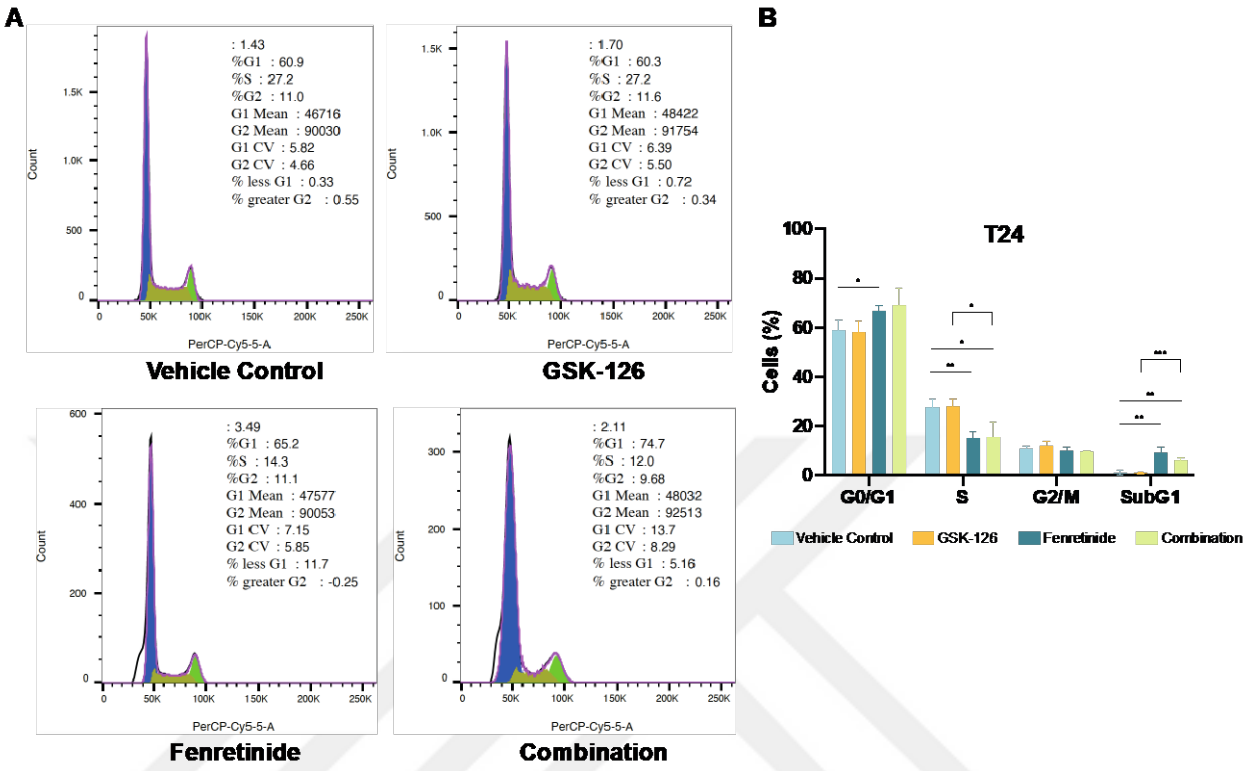


Figure 11. The effects of drug combination on cell cycle profile

A) Representative flow cytometry plots showing the distribution of cells in cell cycle phases following treatment with fenretinide (5 μM, 72 h) and GSK-126 (5 μM, 72 h) alone or in combination. B) Quantitative analysis of the distribution of cells in G0/G1, S, G2/M, and sub-G1 phases of the cell cycle. Data represents mean ± SD between triplicates (two-tailed unpaired t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

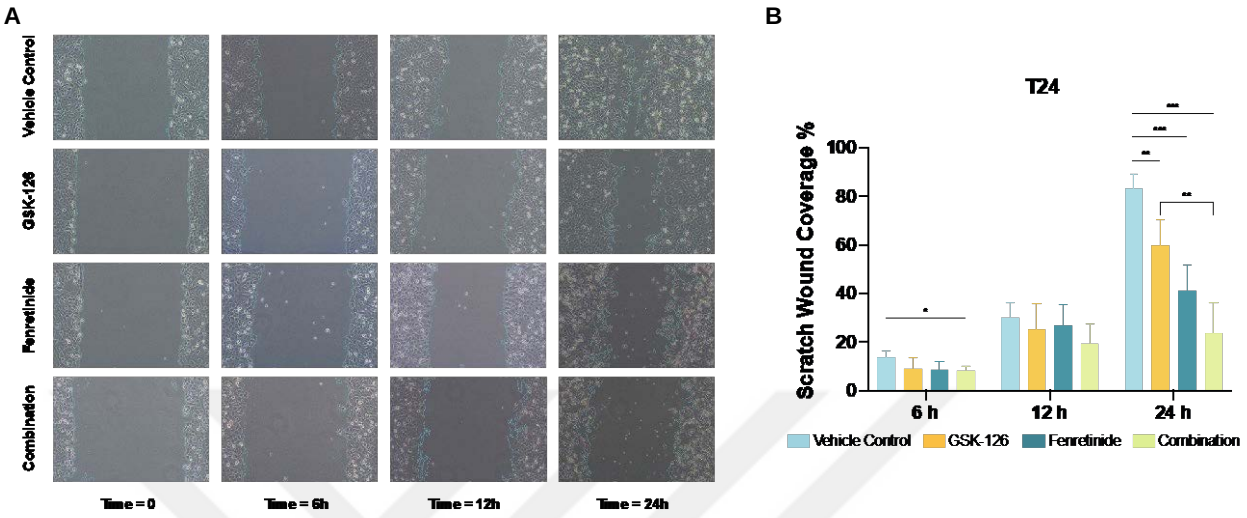


Figure 12. The effects of drug combination on cell migration

(A) Representative images of the scratch assay to measure the migration ability of T24 cells treated with fenretinide (5 μM), GSK-126 (5 μM), or the drug combination at different time points. (B) The quantitative analysis of the scratch assay. Data represents mean ± SD between triplicates (two-tailed unpaired t-test: *p < 0.05, **p < 0.01, ***p < 0.001).

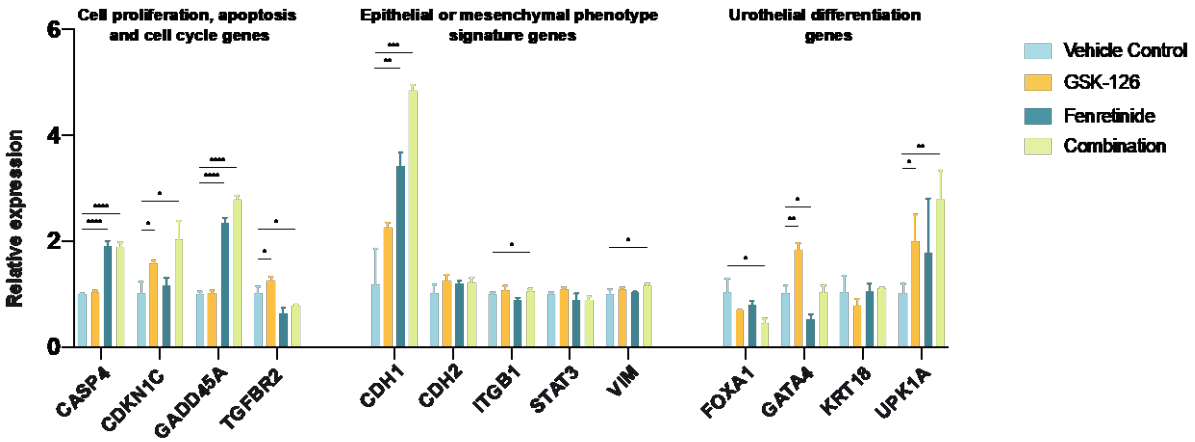


Figure 13. The effects of drug combination on gene expression

RT-qPCR analysis of indicated genes in T24 cells treated with fenretinide and/or GSK-126 (5 μM, 72 h). Gene-specific data were normalized to GAPDH expression and are represented as relative expression compared to vehicle control. Data represents mean ± SD between triplicates (two-tailed unpaired t-test: *p < 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001).

4.4 Single or Combination Treatments Elicit a Divergent Set of Differentially Regulated Genes

To discover the transcriptional programs changed by co-targeting of RA and EZH2 at a genome-wide scale, we performed RNA-sequencing. In our RNA-seq analysis, a total of 4308 common protein-coding genes were mapped and found to be differentially expressed in T24 cells following treatment with fenretinide and GSK-126, either as a single agent or drug combination. Hierarchical clustering analysis identified five groups of differentially expressed genes following single and combinatorial treatments as compared to control (Figure 14A). The gene ontology **(**GO) analysis revealed that the first cluster, strongly downregulated by combination treatment (CoSDR), includes genes related to RNA processing and mitotic spindle organization (Figure 14B). Enrichment analysis of the genes identified in the second cluster, downregulated by fenretinideonly or combination treatments (CoFeDR), is associated with regulating chromatin organization, DNA replication, and relevant processes (Figure 14B). The third cluster, upregulated by GSK-126 treatment (GskUR), consists of the genes involved in developmental processes (Figure 14B). The fourth cluster, upregulated by fenretinide-only or combination treatments (CoFeUR), exhibited enrichment of certain gene signatures, predominately related to apoptosis, unfolded protein response, and the endoplasmic reticulum (ER) stress response pathways (Figure 14B). A limited number of genes were upregulated by fenretinide treatment alone (FeUR, cluster 5), and those genes were not associated with a specific pathway (Figure 14A).

Generally, upon treatment with fenretinide alone or in combination with GSK-126, the expression levels of canonical repressors (E2F3B, E2F5, and E2F6) were considerably increased, whereas the expression levels of the activator transcription factors E2F1 and E2F2 were strongly downregulated (Figure 15). Overall, these analyses revealed that transcriptional signatures are consistent with phenotypic responses, which involve the activation of apoptotic pathways and the attenuation of cell cycle and proliferation processes.

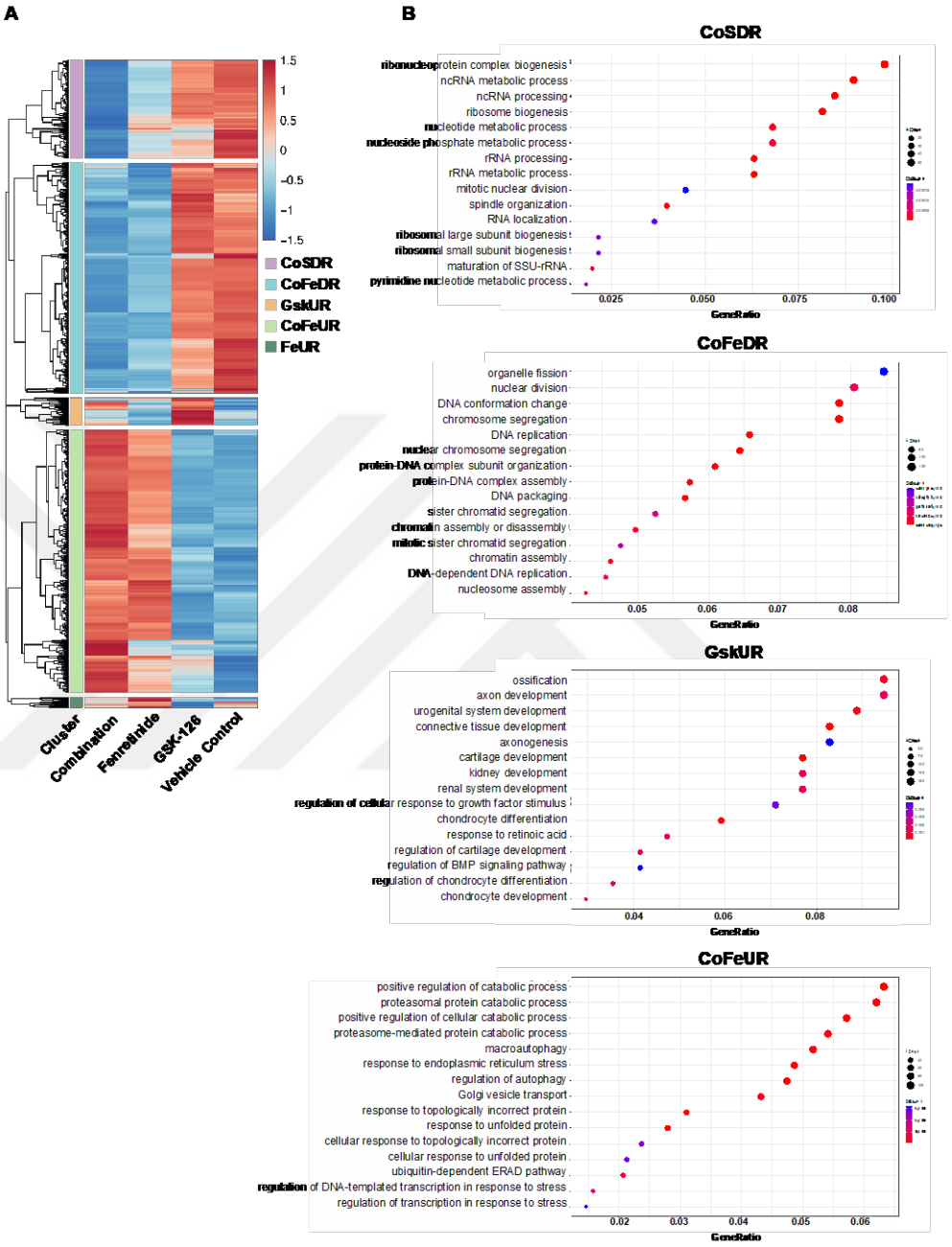


Figure 14. Hierarchical clustering of differentially expressed genes

A) RNA-seq heatmap highlighting hierarchical clustering of differentially expressed genes in T24 cells following single or combinatorial treatments (5 μM fenretinide and/or 5 μM GSK-126 for 72 h) versus control using log2 FC> 0.5 cutoff. Red and blue express high and low expression levels of genes, respectively. B) Gene Ontology analysis illustrating upregulated biological processes for each cluster.

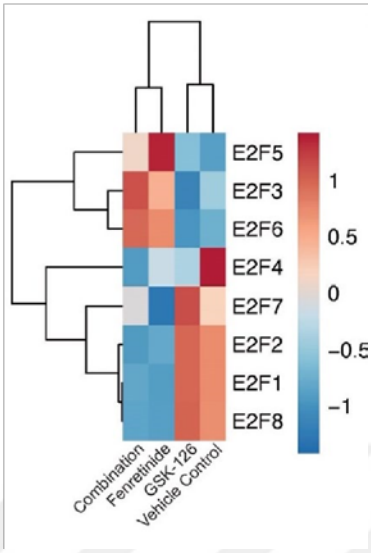


Figure 15. The relationship between fenretinide and combination treatments and cell cycleassociated factors

Heatmap illustrating differentially expressed E2F family genes between control and T 24 cells treated with fenretinide (5 μM, 72 h) and/or GSK-126 (5 μM, 72 h) using log2 FC>0.5 cutoff. High and low gene expression levels are represented by red and blue, respectively.

4.5 Assosiation of Differentially Expressed Genes with H3K27me3 Profiles

We hypothesized that the anti-oncogenic effects might be established through the activation of new transcriptional programs in the absence of EZH2-mediated repression. To test this theory, we carried out ChIP-seq of H3K27me3 on T24 cells. First, we confirmed that GSK-126 results in a global reduction of H3K27me3 without altering EZH2 levels (Figure 16). We characterized the alterations in the H3K27me3 profile induced by drugs at a genome-wide level. As expected, we identified a global decrease in peak binding events across the genome, specifically at the transcriptional start sites of genes in cells treated with GSK-126 alone and in combination with fenretinide (Figure 17). ChIP-seq data demonstrated that the genes belonging to the GskUR cluster have the highest H3K27me3 signal in vehicle control, most likely reflecting the involvement of these genes in developmental functions (Figures 14B and 17C). H3K27me3 profiles at genes belonging to CoSDR, CoFeDR, and FeUR clusters were almost unchanged among the tested conditions (Figures 17A, B, and E). Remarkably, inspection of H3K27me3 profiles at the CoFeUR

cluster reveals that the level of H3K27me3 is even more decreased after combination treatment, especially downstream of transcriptional start sites (TSS), suggesting that fenretinide treatment additionally causes a reduction in H3K27me3 in addition to GSK-126 treatment (Figure 17D). As it was shown by GO term analysis in Figure 14B, CoFeUR genes are involved in ER stress and unfolded protein response (UPR). Additionally, we checked the H3K27me3 profiles of a subset of genes involved in UPR and ER stress and confirmed the decrease in H3K27me3 levels after GSK126 and combination treatments at ATF3, FICD, HSPA9, and PPP2CB genes (Figure 18).

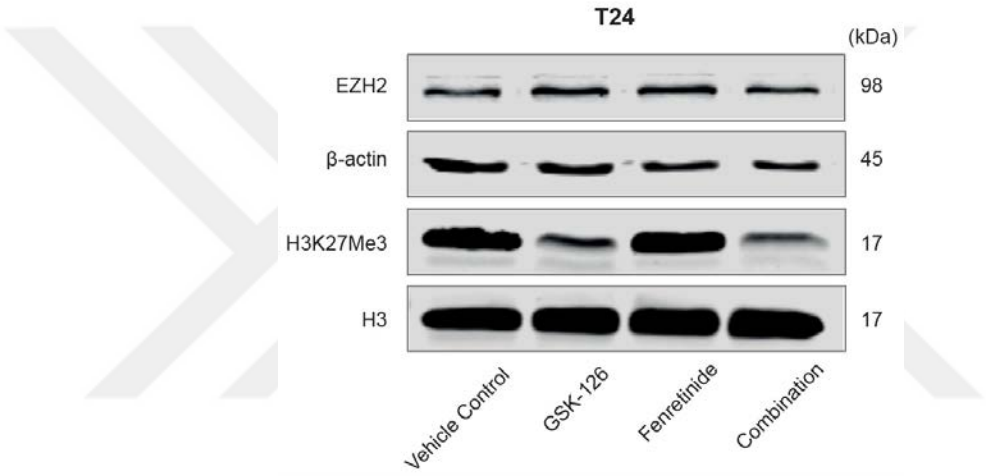


Figure 16. Effects of drugs on EZH2 and H3K27me3 expressions

Western blots performed on T24 cells treated with fenretinide (5 μM, 72 h) and/or GSK-126 (5 μM, 72 h), assessing the expression of EZH2, β-actin, H3K27me3, and H3.

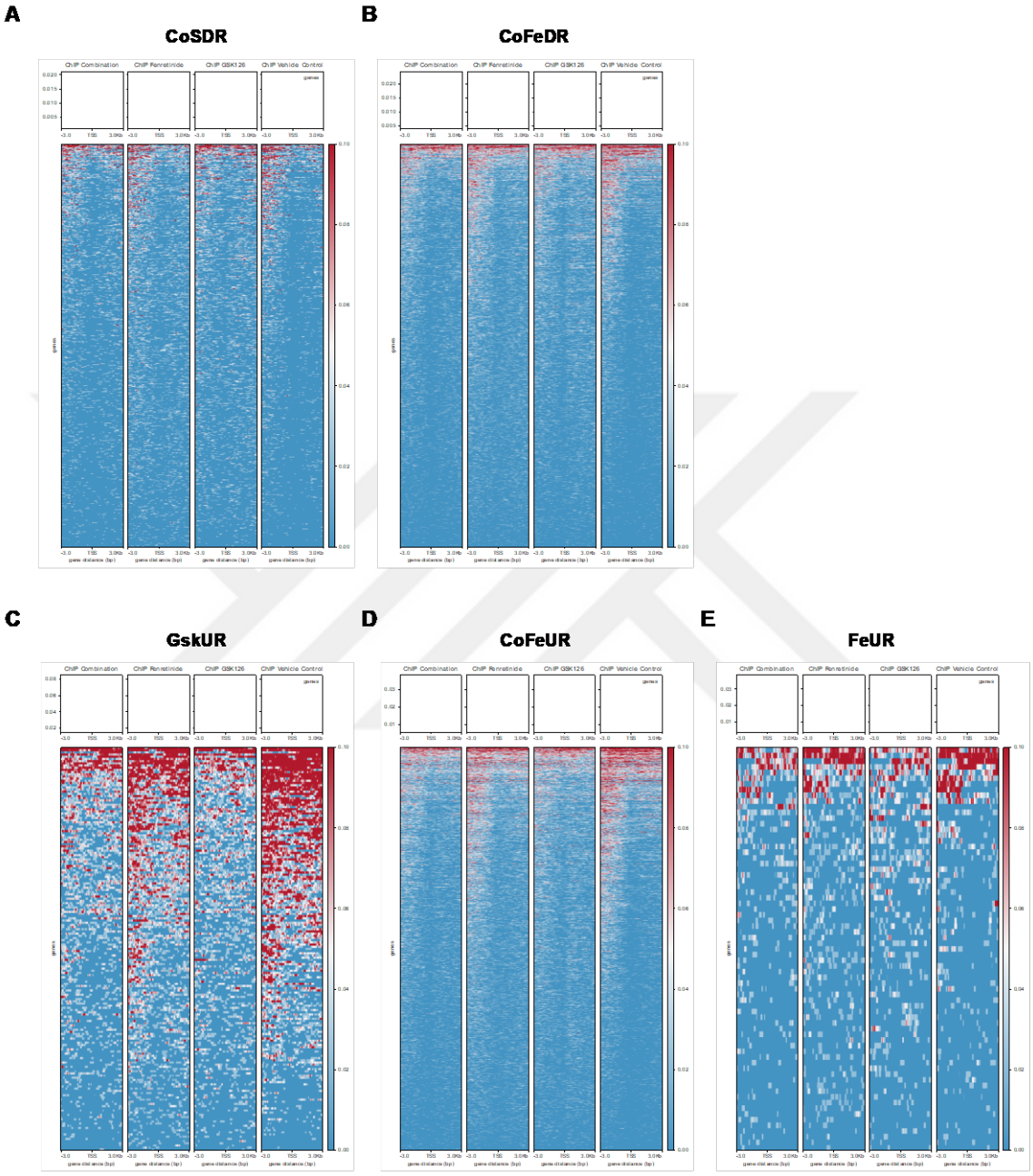


Figure 17. Effects of drugs on global H3K27me3 profiles

Signal intensity heatmaps showing H3K27me3 ChIP-seq genomic regions in ±3.0 kb distance of the transcriptional start sites (TSS) at the genes belonging to CoSDR (A), CoFeDR (B), GskUR (C), CoFeUR (D), and FeUR (E) in T24 cells.

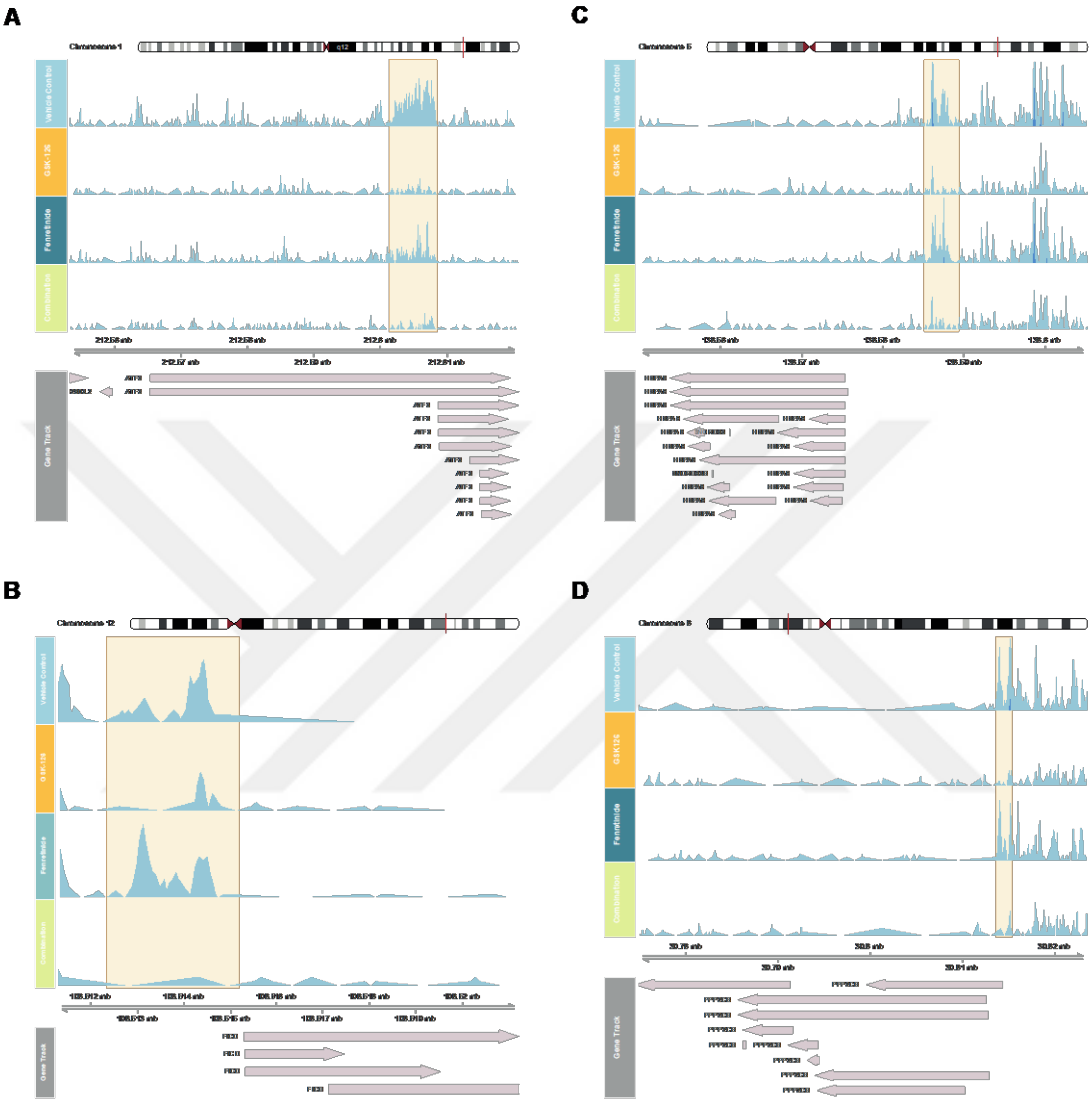


Figure 18. H3K27me3 profiles of genes linked to unfolded protein response pathways

The tracks show H3K27me3 ChIP-seq signals of ATF3 (A), FICD (B), HSPA9 (C), and PPP2CB (D) genes in T24 cells. The signal peak regions are highlighted.

4.6 Differential Transcription Factor Activities Are Linked with Single and Combination Treatments

We proceeded to discover potential regulatory factors involved in controlling distinct groups of differentially expressed genes following single or combination treatments. We investigated the relationship between differentially expressed genes and transcription factor activities using multiple curated databases queried by the EnrichR web interface.

E2F family transcription factors were considerably enriched for the genes associated with the CoSDR and CoFeDR clusters, confirming the downregulation of the genes involved in cell cyclerelated processes in these clusters (Figure 19A, B). We identified the genes belonging to the GskUR cluster as major Polycomb targets, verifying their status as PRC2 target genes (Figure 19C). The genes identified in the CoFeUR cluster were enriched for the C/EBP family (Figure 19D). Among transcription factors regulating the expressions of CoFeUR cluster-member genes, C/EBPβ (CCAAT Enhancer Binding Protein Beta) and CHOP (C/EBP homologous protein, DDIT3) were of particular interest. EnrichR analysis not only identified C/EBPβ and CHOP as one of the top candidate transcription factors, but also transcriptome analysis demonstrated that C/EBPβ and CHOP considerably upregulated following fenretinide-only and combination treatments. The elevated expressions of these transcription factors were also verified at the protein level (Figure 20). For the CoFeUR cluster, enrichment for other transcription factors, including USF2, NFE2, STAT3, and VDR, was not considered significant since trancriptome data did not show a marked change for these factors. Considering that C/EBPβ and CHOP are the transcription factors that can independently regulate their own target genes, we next assembled a set of their target genes derived from the EnrichR database. In contrast to the non-target genes of C/EBPβ and CHOP, the target genes of these factors were found to be significantly upregulated after fenretinide or combination treatments (Figure 21). This finding supports the significance of C/EBPβ and CHOP in the regulation of genes in response to fenretinide and combination treatments. Of note, these transcription factors have a pivotal role in the ER stress response and apoptosis.

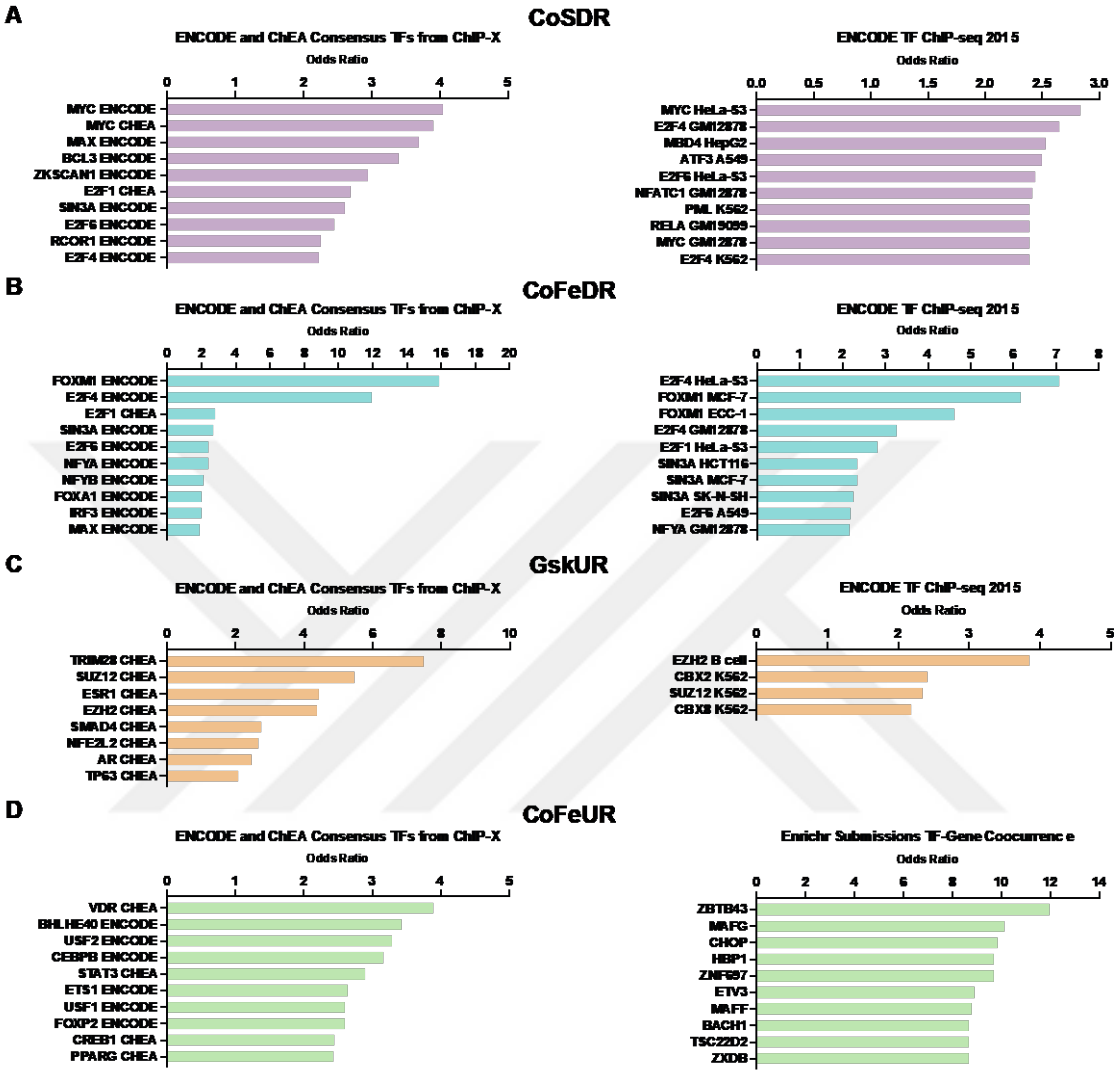


Figure 19. Transcription factor activities of differentially expressed genes

The relationship between differentially expressed genes in the CoSDR (A), CoFeDR (B), GskUR (C), and CoFeUR (D) clusters and transcription factor activities, which were determined using the information available in the EnrichR database. Adjusted P value < 0.05.

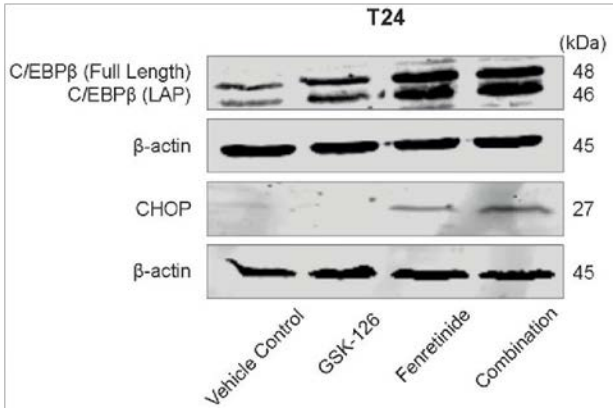


Figure 20. Upregulation of C/EBPβ and CHOP at protein level by fenretinide and combination treatments

Western blot analysis of C/EBPβ and CHOP expression in T24 cells following treatment with fenretinide (5 μM, 72 h), GSK-126 (5 μM, 72 h), or the combination of drugs.

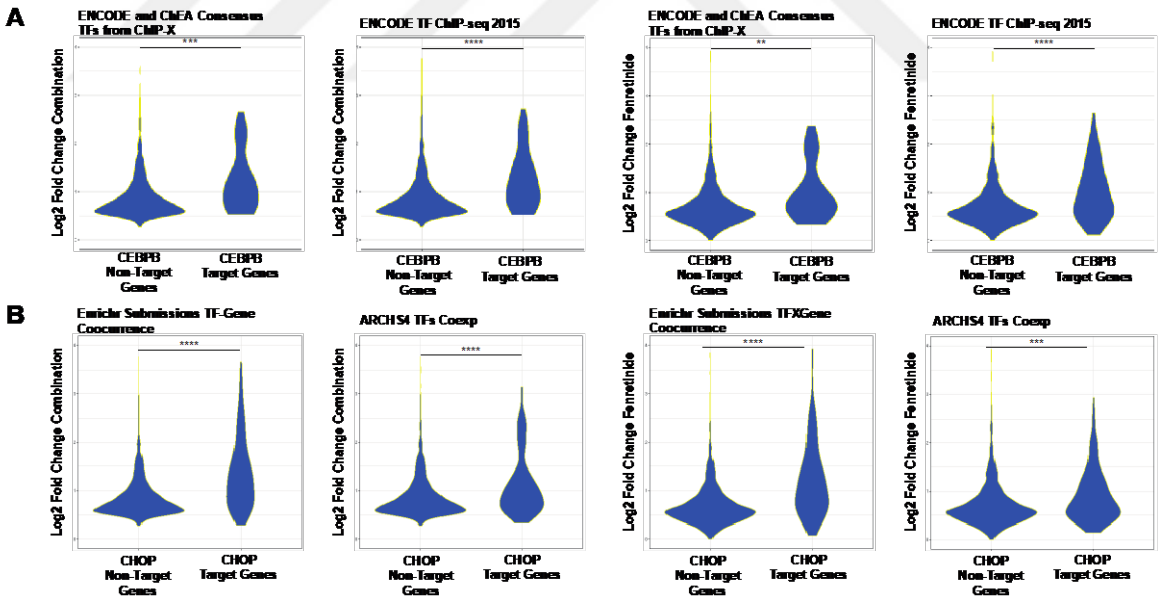


Figure 21. Transcriptional upregulation of putative target genes of C/EBPβ and CHOP after fenretinide or combination treatments

Violin plots of expression level for predicted target and non-target genes for C/EBPβ (A) and CHOP (B) in the CoFeUR cluster, which were determined by data derived from the EnrichR database.

4.7 Identification of EZH2 Target Genes

By classifying the upregulated genes as combination-enhanced and fenretinide-driven, we conducted an elaborate analysis of the CoFeUR cluster (Figure 22A). Gene-concept network analysis revealed that combination-enhanced genes were related to unfolded protein response (UPR), cellular responses to stimuli, and some metabolic processes, consisting of the genes ATF3, DNAJB1, HSPA1A, HSPA1B, and HSPA5 (Figure 22B).

To pinpoint EZH2 target genes, we integrated the gene expression data of the genes that are dominantly combination enhanced and GSK-126 upregulated with literature [12]. We considered that genes identified as enhanced by the combination compared to fenretinide-driven genes may be transcriptionally upregulated by effects facilitated by GSK-126. To investigate the hypothesis that these genes are epigenetically repressed by PRC2 activity, we performed H3K27me3 ChIP for these genes. Remarkably, ChIP-qPCR analysis revealed that GSK-126 treatment significantly decreased H3K27me3 levels for AKR1C2, ATF3, HSPB8, IGFBP3, MEIS3, NDRG1, PTGS2, and SERPINE2 genes (Figure 23A). Moreover, ChIP-seq analyses validated ChIP-qPCR analyses (Figure 23B). Having established the role of GSK-126 in driving the UPR signature in stimulated T24 cells, we next investigated the changes in a subset of predicted target genes to verify whether EZH2 inhibition causes derepression of these genes in MIBC cell lines. Inhibition of EZH2 by GSK-126 or combination treatments significantly increased the RNA expression levels of these genes in 5637 cells as well (Figure 24). Therefore, our results establish a correlation between an increase in gene expression and a decrease in H3K27me3.

Collectively, our data confirmed that AKR1C2, ATF3, HSPB8, IGFBP3, MEIS3, NDRG1, PTGS2, and SERPINE2 genes are directly targeted by EZH2-mediated H3K27me3 in MIBC, supported by H3K27me3-driven robust changes in gene expression signature. Our hypothesis is that EZH2 silences ER stress and UPR-mediated anti-oncogenic transcriptional programs. Simultaneous effects of retinoic acid pathway activation and EZH2 inhibition may trigger these pathways.

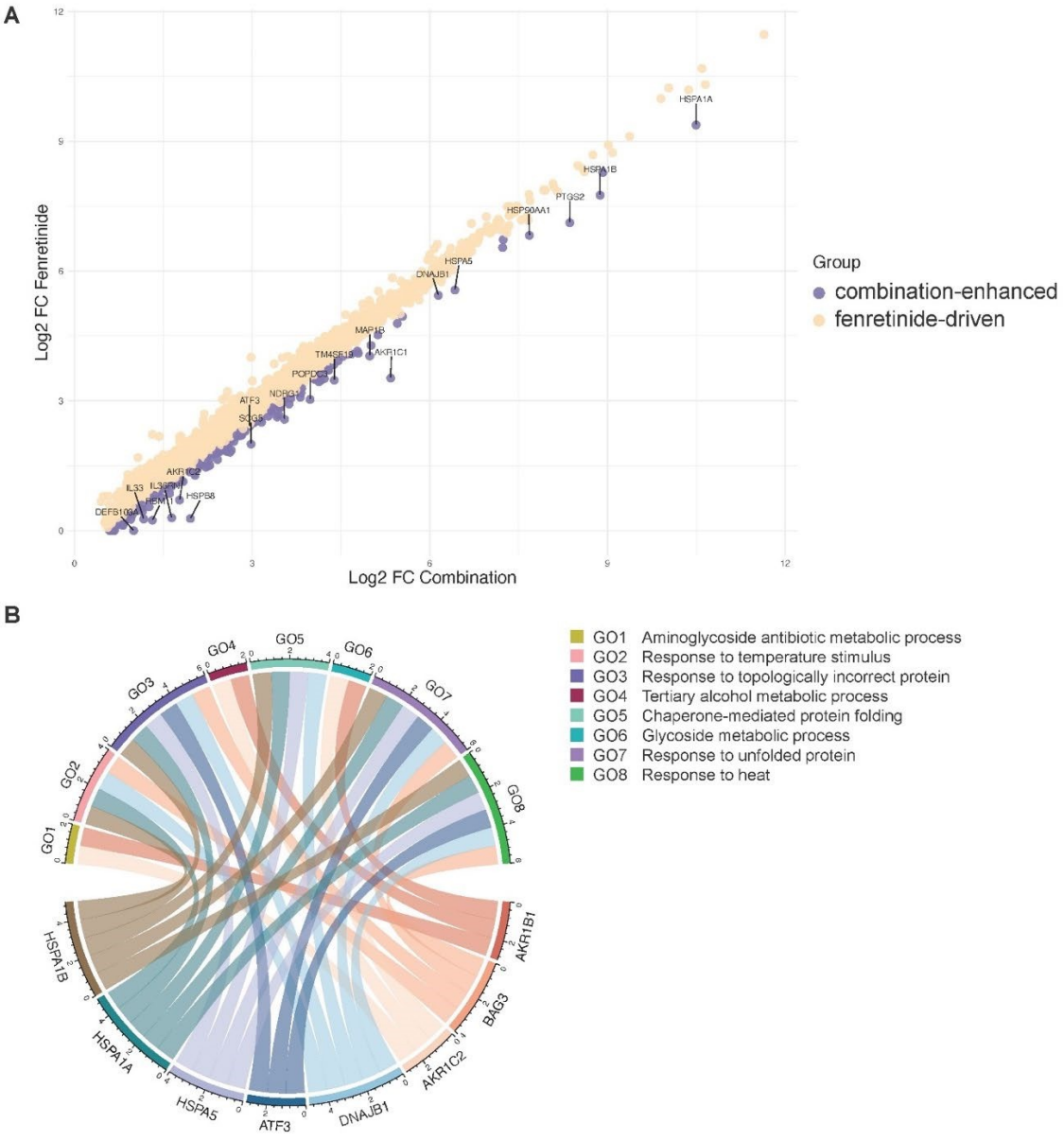


Figure 22. Identification of targets repressed by EZH2

A) Identification of combination-enhanced genes in the CoFeUR cluster. B) Chord diagram of potential EZH2 target genes and their associated GO biological terms.

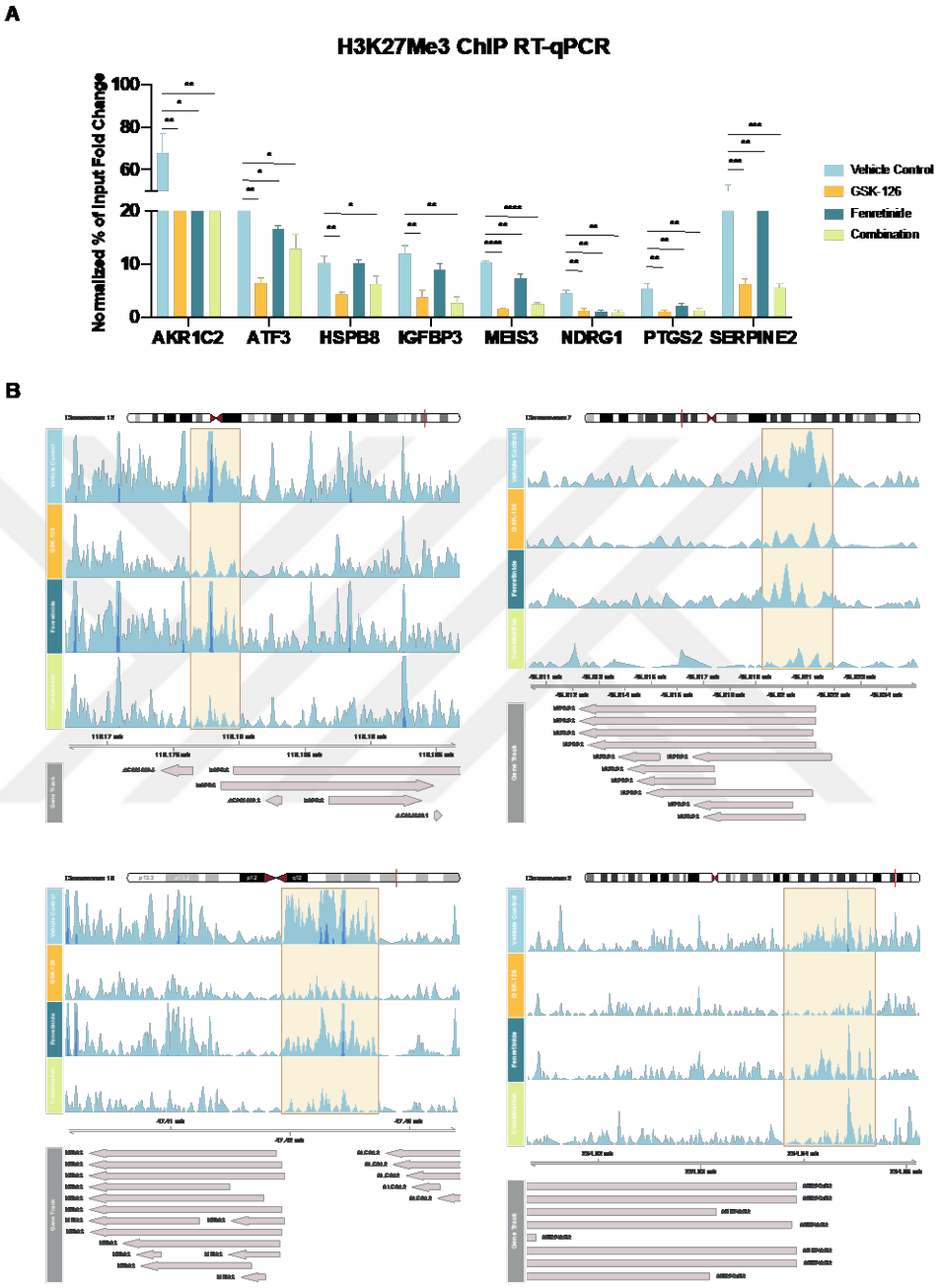


Figure 23. Differential H3K27me3 levels of predicted EZH2 target genes

A) RT-qPCR analysis of chromatin immunoprecipitation of H3K27me3 at canonical EZH2 targets in T24 cells treated with GSK-126 (5 μM, 72 h), fenretinide (5 μM, 72 h), or their combination. The data were normalized to the negative control, GLIS2. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, two-tailed *t*test. B) Genomic tracks of a subset of identified EZH2 target genes (HSPB8, IGFBP3, MEIS3, and SERPINE2) showing H3K27me3 ChIP-seq signals in T24 cells.

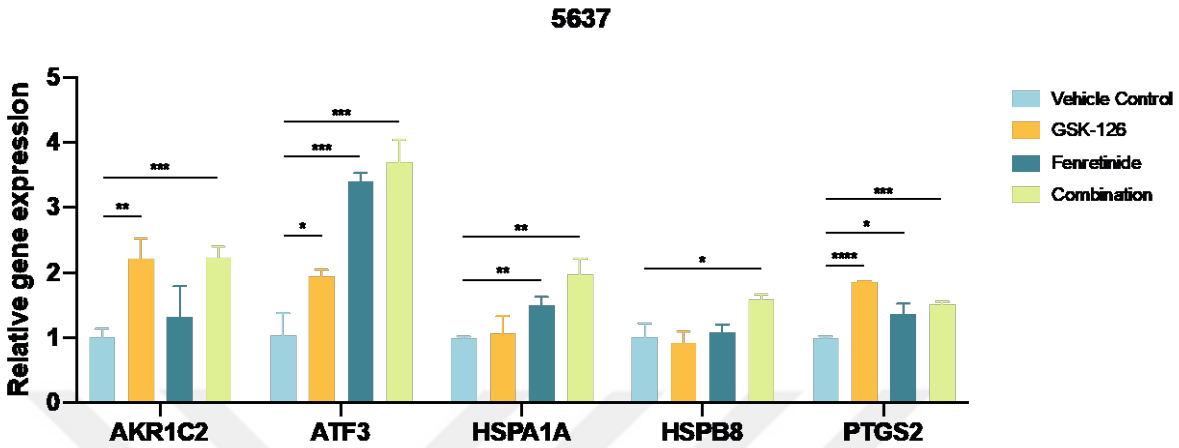


Figure 24. Differential expression levels of a subset of predicted EZH2 target genes induced by drugs

The expression levels of a subset of predicted target genes linked to the unfolded protein response in 5637 cells treated with fenretinide (5 µM, 72 h), GSK-126 (5 µM, 72 h), or their combination. Data were normalized to GAPDH expression and represented as relative expression compared to vehicle control. Data are presented as mean ± SD. Two-tailed unpaired t-test: *p< 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001.

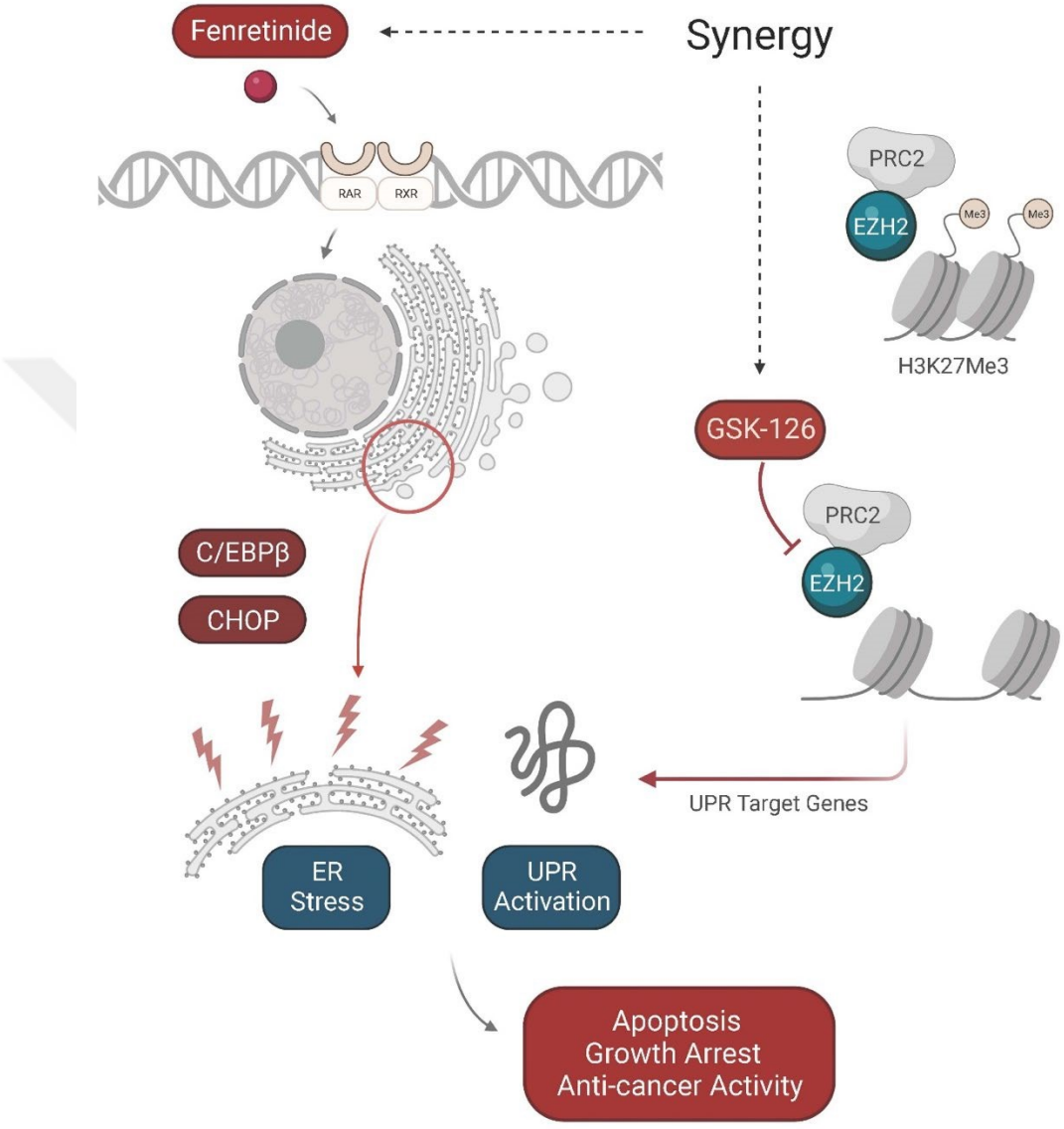


Figure 25. Proposed model highlighting the mechanisms of co-targeting of EZH2 and RA pathway in bladder cancer

A proposed schematic model illustrates the synergistic interaction of EZH2 methyltransferase activity inhibition and retinoic acid pathway activation, leading to C/EBPβ and CHOP-dependent activation of apoptotic programs and unfolded protein response.

5 DISCUSSION

Bladder cancer patients exhibit an elevated recurrence rate and a minor advantage from chemotherapy, primarily as a consequence of untailored therapeutic approaches, substantial tumor heterogeneity, and dynamic cellular plasticity. Additionally, the majority of monotherapies have limited efficacy and promote drug resistance. Thus, there is an urgent need to provide novel therapy options, which is critical for bladder cancer patients.

In this research, we aim to utilize the actions of the RA pathway and EZH2 inhibition combinatorially and hence potentiate the therapeutic response in bladder cancer. This study provides evidence that targeting the RA/EZH2 axis represents a promising strategy for bladder cancer. This phenomenon correlates with previous studies illustrating the significance of targeting PRC2 and RA signaling simultaneously in a variety of cancers. For instance, according to research by Villa and colleagues, most of the PRC2-induced epigenetic silencing programs can be reversed by RA [90]. Recently, Benoit and colleagues demonstrated that PRC2 inhibition could potentially overcome RA resistance [91]. The data demonstrate that the combined actions of EZH2 inhibition and retinoids synergistically inhibit bladder cancer proliferation and stimulate the expression of marker genes linked to apoptosis, cell cycle arrest, epithelial phenotype, and urothelial differentiation.

Despite the progress in the understanding of the anti-oncogenic effects of retinoids, the downstream targets driving their biological effects are still poorly understood. Here, we revealed that fenretinide and GSK-126 combination treatment stimulates the expression of C/EBPβ and CHOP, thereby triggering apoptotic reprogramming in bladder cancer cells. Consistent with the prior research, our findings further prove that C/EBPβ and CHOP transcription factors perform their anti-oncogenic roles by promoting apoptosis and the ER stress response [92, 93]. Previous investigations have identified C/EBPβ and CHOP as prominent retinoic acid target genes involved in cell differentiation [94, 95]. Our findings, highlighting C/EBPβ-mediated transcriptional programs as a regulatory node for the anti-oncogenic actions of fenretinide, reinforce this conception. Another important aspect of our results is the positioning of CHOP as a crucial apoptotic regulator

in MIBC. Typically, CHOP is triggered by unfolded protein response and integrated stress response and therefore serves as a primary regulator of ER stress-induced apoptosis [96].

Our hypothesis is that stimulation of anti-oncogenic transcriptional programs driven by C/EBPβ and CHOP is boosted by alterations in chromatin architecture driven by EZH2 inhibition. We speculate that the role of EZH2 inhibition in reassembling accessible chromatin profiles in MIBC cells, thus conducting apoptotic transcriptional reorientation, underlies this synergistic interaction. Previous research has revealed that disruption of PRC2 function can stimulate apoptosis [97]. We further designate C/EBPβ and CHOP as downstream targets of the RA pathway, which stimulate a molecular network enabling apoptotic reprogramming, although we have not elucidated the exact relationship between them. The outcomes of EZH2 inhibition-mediated transcriptional rerouting of C/EBPβ or CHOP accompanied by retinoid activity result in enhanced apoptosis and may thereby make the cytotoxic response more robust in bladder cancer. We also attribute the anti-oncogenic characteristics of this combination to cell cycle arrest, reflecting by the repression of the E2F1 and E2F2 family transcription factors involved in cell cycle-related processes. Recently, Lee and colleagues have identified E2F1 as a putative regulator of EZH2 and a pivotal factor in the progression of bladder cancer [98]. After combination treatment, we detected elevated levels of E2F3B, E2F5, and E2F6 canonical repressors of the cell cycle, temporarily switching off the E2F program in G2 [99]. In support of our findings, the Duprez group revealed the key function of the E2F/EZH2 axis underlying RA resistance [100]. Inhibiting EZH2 activity resulted in an increase in E2F7 and E2F8 repressors in our study. As previously reported, the expression of EZH2, which serves as a critical downstream regulator of the pRB-E2F pathway, can be induced by the E2F family [42].

RNA-seq analyses and subsequent validation enabled us to identify a subset of genes as PRC2 targets. Our results mapping the EZH2 target genes uncovered that these genes were identified in the clusters as primarily functioning in response to topologically incorrect proteins, chaperone-mediated protein folding, and some metabolic processes predominantly associated with UPR function. Underpinning our observations, Arbuckle and colleagues reported that EZH2 inhibition

elevates the expression of inflammatory cytokines and mediators, including PTGS2, and transcription factors involved in the ER stress response, such as C/EBPβ, ATF4, and CHOP [101]. Supporting the previous research by other groups, HSPB8, NDRG1, IGFBP3, PTGS2, and SERPINE2 cellular stress response genes were identified as EZH2 targets in our study [102, 103].

Previous preclinical and clinical trials demonstrated the superior efficacy and reduced therapeutic recurrence of EZH2 inhibitors, especially in lymphomas, but limited responses in solid tumors [104]. The general increase in EZH2 inhibitor efficacy in hematologic compared with solid cancers is attributed to the presence of mutations that result in catalytically hyperactive EZH2, rendering them sensitive to treatment with EZH2 inhibitors. The highest combination efficiency observed in T24 cells may be associated with the presence of KDM6A mutations. Cancers with loss-of-function mutations in the SWI/SNF complex and KDM6A have been reported to be sensitive to EZH2 treatment [47]. Ler and colleagues revealed that KDM6A mutations cause prolonged repression of PRC2-regulated genes in urothelial carcinoma, remarking the therapeutic advantage of EZH2 inhibition in KDM6A-null bladder cancers [12].

Collectively, our research provides novel evidence showing that EZH2 inhibitors cooperate with retinoids to activate a network of genes that enable cytotoxic effects in bladder cancer. Remarkably, the network of regulatory factors recruited by RA-EZH2 inhibition cooperation plays a major role in the positive regulation of cellular catabolic processes, response to ER stress, and topologically incorrect or unfolded proteins, as well as the regulation of DNA-templated transcription in response to stress. These co-regulated targets support the idea that this collaboration favors an anti-oncogenic profile. We hypothesize that EZH2 inhibition acts as a “second strike” that potentiates the anti-oncogenic activities of retinoids. Consequently, supporting apoptotic networks could provide a molecular explanation for the synergistic effects of RA gain and PRC2 loss. Ultimately, our research reveals an entirely novel aspect of the RA/EZH2 partnership.

6 CONCLUSIONS

Overall, our findings suggest a concerted mechanism between retinoic acid pathway activation and EZH2 inhibition for eliciting synergistic effects in terms of the reflection of the antioncogenic profile. The molecular mechanism and the key mediators of anti-oncogenic transcriptional reprogramming by our dual targeting approach were depicted in this study. Our findings reflect the importance of integrating the targeting of the retinoic acid pathway into EZH2mediated changes in the epigenetic landscape. Consequently, our combination concept appears to be a rational strategy to enhance the therapeutic efficacy of bladder cancer and contribute to targeted treatment paradigms.

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8 APPENDIX

8.1 Published Review Article

Name of the article: Retinoic acid signaling and bladder cancer: Epigenetic deregulation, therapy and beyond

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REVIEW

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Retinoic acid signaling and bladder cancer: Epigenetic deregulation, therapy and beyond

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Abstract

Retinoic acid (RA) signaling is a crucial developmental pathway involved in urothelium development, differentiation and regeneration. Deregulation of the RA signaling is highly implicated in several cancers, including bladder cancer, underlying the need to unravel the complete regulatory aspects of the retinoids in bladder tumorigenesis. Given the fact that RA receptors are transcription factors functioning at the chromatin level and act in close cooperation with chromatin modifiers, it is known that retinoids show their efficacy by changing the epigenome. Bladder cancer can be defined as a "disease of chromatin" with mutations identified in the genes involved in chromatin regulation in 80% of the patients. Therefore, a careful examination of the epigenetic backgrounds and the breakdown of the emerging and highly underexplored field of RA dependent regulation of the epigenome is essential to fully understand the retinoid-dependent effects on bladder cancer. With this motivation, in this review, we evaluate the role of RA signaling in bladder cancer with a focus on the regulatory and mutational aspects, emphasizing the deregulatory characteristics in bladder cancer and highlighting the potential treatment opportunities with the RA and derivatives alone or in combination with epigenetic drugs.

KEYWORDS

bladder cancer, epigenetic deregulation, retinoic acid

1 | INTRODUCTION

Retinoic acid (RA), the primary bioactive metabolite of vitamin A, induces a wide-ranging of pleiotropic effects in cell growth and differentiation which are related to embryonic development and adult physiology. Retinoids have crucial functions in several aspects of human physiology including fertilization, morphogenesis, organogenesis, as well as fetal and perinatal development. For adult life, they also modulate reproduction, immunity and vision, and are essential for the proper function of skin, lung, liver and neuronal system.[1]

The RA action is mediated principally by the retinoic acid receptor (RAR) subfamily which acts as transcription factors that modulate the expression of genes. This process is orchestrated by co-activators and co-repressors that alter the chromatin structure and thereby affect the accessibility to DNA.[2-5]

The disturbance of RA signaling pathways underlies the etiology of a variety of malignancies, including leukemias, skin cancer, head/neck cancer, lung cancer, breast cancer, ovarian cancer, prostate cancer, renal cell carcinoma, pancreatic cancer, liver cancer, glioblastoma,

Abbreviations: 4-HPR, N-4-hydroxyphenyl-retinamide; APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; BCG, Bacillus calmette guerin; DBD, DNA binding domain; FDA, Food and Drug Administration; FOX, the human forkhead box; H3K27me3, histone H3 lysine 27 trimethylation; H3K4me3, histone H3 lysine 4 trimethylation; HATs, histone acetyltransferases; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitors; IGFBP, insulin-like growth factor-binding protein; KAT, lysine acetyltransferase; KDM, lysine demethylases; LBD, ligand-binding domain; LSD1, lysine-specific demethylase 1; MIBC, muscle-invasive bladder cancer; NCOA1, nuclear receptor co-activator 1; NCOA2, nuclear receptor co-activator 2; NCOA3, nuclear receptor co-activator 3; NCOR1, nuclear receptor co-repressor 1; NCOR2, nuclear receptor co-repressor 2; NMIBC, nonmuscle invasive bladder cancer; PPARγ, peroxisome proliferator-activated receptor-gamma; PRC2, polycomb repressive complex 2; RA, retinoic acid; RAR, retinoic acid receptor; RAREs, retinoic acid response elements; RXR, retinoid X (or rexinoid) receptor; TDG, thymine DNA glycosylase; UCC, urothelial cell carcinoma.

neuroblastoma and bladder cancer.[1] Furthermore, RA and its derivatives (retinoids) come into prominence as potential chemotherapeutic or chemopreventive agents owing to their effects on differentiation, proliferation and apoptosis.

Bladder cancer is the fifth most common cancer and causes more than 150 000 deaths each year.[6] Recent genomic studies demonstrated the molecular characteristics of the bladder cancer and revealed the mutational landscape in this tumor.[7,8] Excessively high mutational load of the epigenetic machinery in bladder cancer points out to the aberrant regulation of the epigenome in this tumor. Considering that the RARs act at the chromatin level in close cooperation with epigenetic factors, it is obvious that retinoids exhibit their physiologic effects mostly via the modulation of the epigenome. Therefore, a comprehensive understanding of retinoid-dependent effects on bladder cancer and unleashing the therapeutic potentials of retinoids in bladder cancer require focusing on RA-regulated epigenetic networks.

The crucial role of RA signaling pathway in urothelial development, differentiation, homeostasis and regeneration, as well as potential tumorigenic consequences of the deregulation of this signaling pathway in bladder carcinogenesis are well documented.[9-11] Nevertheless, the field which combines the regulatory and mutational aspects of RA signaling in bladder cancer in connection to epigenetic regulation is largely unexplored. In this review, we thoroughly revise the literature about the role of RA signaling in bladder cancer development in connection to transcriptional and epigenetic deregulation and RA-signaling associated genomic mutations in bladder cancer.

2 | RA SIGNALING AND ITS TRANSCRIPTIONAL REGULATION

Dietary-derived Vitamin A which is a crucial nutrient for adult health and embryonic development is metabolized intracellularly to generate its active metabolites such as 11-*cis*-retinoic acid and all-*trans*-retinoic acid (ATRA; generally regarding as RA). In virtually every cell and organ system, retinoids perform a broad variety of essential and diverse functions, most particularly in terms of vision, cell proliferation, differentiation of the cells, immune function, neural function and the constitution of the body form in early development.[5]

RARs have been identified as the members of the steroid/thyroid hormone nuclear receptors superfamily operating principally as ligand-inducible transcription factors. RA-regulated gene expression has a highly dynamic nature, governed by corepressor and coactivator complexes.[2] Retinoid X (or rexinoid) receptors (RXRs) act as imperative heterodimerization partners for RARs, likewise heterodimerize with other nuclear receptors. For this reason, RXR ligands potentially affect the signaling of other pathways.[12] To date, six distinct RARs and RXRs, titled as RARα, RARβ and RARγ as well as RXRα, RXRβ and RXRγ have been described, each encoding multiple N-terminal protein isoforms. These isoforms result from splicing variations or the use of different promoters, accordingly diverge in their promoters and A region sequences. However, these isoforms have identical B-F regions which comprise DNA-binding domains (DBD) and ligand-binding domains (LBD) important for dimerization, trans-activation and co-repressor interaction.[13]

RA modulates gene transcription by inducing epigenetic changes via RARs which bind to specific genomic DNA sequences known as RA response elements (RAREs).[14] Similar to other important developmental regulators, RARs have the ability to act as a molecular switch. In the absence of ligand, RAR-RXR heterodimers associate with co-repressors such as nuclear receptor co-repressor 1 (NCOR1) and NCOR2 (also known as SMRT), which in turn recruit histone deacetylase (HDAC) protein complexes and Polycomb repressive complex 2 (PRC2). This process eventuates with histone H3 lysine 27 trimethylation (H3K27me3), chromatin condensation and gene silencing.[3,14] Conversely, upon RA binding to RAR-RXR, this binding stimulates conformational alterations in the DBD of these receptors, releasing the corepressors and creating a novel interaction surface for coactivator complexes.[15] Recruited co-activators such as nuclear receptor co-activator 1 (NCOA1; also known as SRC1), NCOA2 (also known as SRC2) or NCOA3 (also known as SRC3) in turn recruit histone acetyltransferases (HATs) and Trithorax proteins, which regulate histone H3 lysine 4 trimethylation (H3K4me3) levels, decompress chromatin and activate the transcription machinery.[2,3] Co-activators and co-repressors which regulate receptor activity have an excessively invertible nature, as evidenced by fast dissociation of co-repressor proteins in response to RA[16,17] and re-association upon RA removal.[14,18]

3 | IMPORTANCE OF RA DEPENDENT GENE REGULATION IN UROTHELIUM DEVELOPMENT

RA has been demonstrated to have a key role in self-renewal, pluripotency and specification of embryonic stem cells and other progenitor cells. In this respect, many studies have proved the role of RA signaling in urothelial specification, homeostasis and regeneration.[9-11] RA is synthesized in the stromal region, a domain substantial for modulating urothelial maintenance and regeneration through Wnt, Bmp and Shh signalization and essential for mediating RAR signaling in the embryonic and adult urothelium. RA and Wnt signaling govern urothelial regeneration by regulating genes such as Bmp.[11] In addition, retinoid signaling pathways have been involved in the development of bladder from urogenital sinus[19] and differentiation of the ureteric mesenchyme.[20,21] Gandhi et al demonstrated that RA signaling controls the maintenance of the adult urothelium in a stable state and modulates the transition between maintenance and renewal.[9] Further, it was demonstrated that RA promotes embryonic stem cells to differentiate into urothelial cells, in vitro.[10]

Several transcription factors are implicated in RA signaling-dependent urothelium development. Mauney et al demonstrated that particular GATA transcription factors are fundamental downstream regulators of RA-mediated uroplakin expression.[10] GATA4 and GATA6 which are transcriptional targets of RARγ[22,23] and RARβ2[24]

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signaling complexes, respectively, contribute to the progression of certain urogenital epithelial lineage and eventually have a substantial impact on bladder specification and maturation. Moreover, Peroxisome proliferator-activated receptor-gamma (PPARγ) which requires hetero-dimerization with RXRα for activation,[25] has been reported as an important activating signaling molecule for urothelial differentiation process.[26-28] It has been suggested that the loss of PPARγ could be a notable incident in bladder cancer progression.[29] Besides, emerging evidence indicates the central role of The human forkhead box (FOX) family transcription factors in urothelial development, differentiation and malignancy.[16,30,31] The RA-specific effects on the expression and the activity of certain FOX transcription factors were revealed by a number of studies. RA has been shown to reduce the expression of FOXM1 in oral squamous cell carcinoma,[32] neuroblastoma[33] and ovarian cancer[34] cells, largely affecting the proliferative characteristics of these cells. In addition, RA was shown to induce the expression of *Foxa1* and *Foxa2*, critical for the endoderm differentiation gene expression program[35] and RA-induced upregulation of *Foxa1* and *Foxa2* was shown to accompany the expression of urothelial marker genes[10] in mouse embryonic stem cells. It was also demonstrated that although FOXA1 is robustly expressed in non-muscle invasive bladder cancer (NMIBC), the expression of FOXA1 is considerably reduced in muscle-invasive bladder cancer (MIBC), as well as in high grade which means poorly differentiated tumors.[36] In summary, the RAR/PPARG/RXRA FOXA1/GATA cycle pointedly involves in the differentiation of the normal urothelium.

4 | DEREGULATION OF RA SIGNALING IN BLADDER CANCER

Contrary to the mutations in RARα inherent in acute promyelocytic leukemia (APL), mutations in RARs are not common. Nevertheless, the retinoid signaling pathway is frequently disrupted in malignant cells. Therefore, this situation provokes the deprivation of retinoid responsiveness in tumor cells.[5]

The substantial role of RA in urothelium development, differentiation and regeneration raises the need for research investigating the association of RA with the emergence of urothelial malignancies. Indeed, a number of lines of evidence points to the dysregulation of retinoid pathways in the pathogenesis of urothelial malignancies including bladder cancer. The role of RA in regeneration and differentiation of urothelial cells, in part through activation of transcription factors like FOXA1 is well established. In line with that, it was demonstrated that Vitamin A deficiency in mice caused squamous metaplasia to occur in the epithelium of the urinary tract.[37,38] Moreover, genetic loss-of-function studies in mouse by Ghandi et al further demonstrated that differentiation of intermediate and superficial cells in embryogenesis, and regeneration of the superficial cell layer upon injury depend on an unimpaired and well-functioning RA signaling pathway.[9] This phenomenon indicates the crucial role of RA signaling for maintenance of a differentiated phenotype in mature urothelium and potentially preventing neoplastic transformation.

Among the RARs (α, β and γ), RARβ2 is the most ascendant receptor, regulating the inhibitory activities of RA on cell proliferation.[39] The RARβ2 gene is constantly lost or epigenetically silenced in human cancers,[40,41] which is generally linked with the retinoid resistance. Furthermore, clinical proofs suggest that expression of RARβ2 is in general diminished with the tumor grade.[39] Remarkably, restoration of RARβ2 expression reactivates RA-dependent growth repression.[41] RARβ has elevated methylation frequencies in human bladder cancers (ranging from 15% to 93%).[42-46] It was demonstrated that the frequencies of methylation at the promoter regions of RARβ2 in normal and carcinoma in situ samples from urothelial cell carcinoma (UCC) patients correlated with disease progression.[47] It was also shown that RARβ mRNA levels are altered in bladder cancer.[48,49] In addition to the alterations determined in the regulation of RARβ, recurrent *RXRA* mutations are observed in 5% to 8% of human bladder cancer[50] and a G to T point mutation was found at position 60, in highly conserved region of the hRARα gene, in the immortalized urothelial cell line, HUC-BC.[51]

The many lines of evidence linking the dysregulation of RA signaling with bladder cancer development strongly suggest that developing novel RA analogs to trigger differentiation and induce apoptotic pathways in urothelium will be a fruitful pursuit.[21]

5 | APPLICATIONS OF RETINOIDS IN BLADDER CANCER

ATRA has captivated researchers for decades with its potential efficacy for chemoprevention and treating human cancers including breast, ovarian, renal, head and neck, melanoma, leukemias and prostate cancers.[52]

There has been plenty of evidence that retinoids inhibit cell-cycle progression in various human cancer cells by modulating cyclins, CDKs and cell cycle inhibitors through different signaling pathways.[40] RA usually induces a cell cycle block in the G1 phase with an increase in the proportion of the cells which are in G0/G1 phase and a decline in the proportion of the cells which are in the S phase.[5] Accumulated evidence support that retinoids induce the expression of cell-cycle regulator p21WAF1/CIP1 and tumor-cell-selective apoptosis ligand TRAIL, and represses AP1 in chemical carcinogenesis.[12,53] Furthermore, retinoids are main compounds in differentiation therapy. Typically, differentiation therapy aims to boost the endogenous differentiation process of the malignant cells to revert the tumor phenotype. Deficient or aberrant differentiation observed in numerous cancers occurs due to several alterations that disrupt signaling pathways, epigenetic modifiers and transcription factors, preventing the proper expression of the genes required for differentiation. Terminal differentiation elicits the deprivation of long-term proliferative potential and ultimately induction of apoptosis. ATRAs ability to compel disrupted maturation programs to be completed underlies the principle of differentiation therapy. Routine and effective clinical use of ATRA has been so far shown to treat APL and precancerous lesions such as leukoplakia, actinic keratosis and cervical dysplasia.[13]

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Considering the importance of the retinoid signaling pathway in bladder carcinogenesis, revival of retinoid function could be an effectual therapeutic strategy for bladder cancer prevention and/or treatment (Table 1). Chemopreventive activities of retinoids/retinoid analogs are supported by strong experimental evidence and corroborated by rat and murine bladder carcinogenesis models, presumedly by readjustment of the normal differentiation status of premalignant and malignant cells.[55] It was shown that ATRA resulted in dose-dependent reduction in cell growth in bladder cancer cell lines.[57] Zou et al showed that, in human bladder cancer cell lines, retinoid analog N-4-hydroxyphenyl-retinamide (Fenretinide or 4-HPR) treatment had resulted in apoptosis, G1 cell cycle arrest and decrease in cell growth.[56] Furthermore, treatment with retinoid analogs resulted in remarkable reduction in the formation of premalignant and malignant lesions in bladder epithelium of carcinogen-induced tumors in rats.[58-60] It was also shown that in vitro normal human urothelial cells in the absence of exogenous retinoids possess a squamous phenotype, but revert to a basal phenotype upon treatment with RA derivatives.[54] These observations highlight the potential impacts of developing therapeutic agents targeting RA signaling pathway to increase the efficacies of current treatments used in bladder cancer.

Since the long-term administration of natural retinoids is largely restricted by vitamin A toxicity, such as liver toxicity, teratogenicity, bone and connective tissue damage, synthetic retinoids are generally preferred for clinical use. The synthetic retinoid Fenretinide (also titled as 4-HPR), an analog of ATRA, preferentially accumulates in bladder tissue. Fenretinide has been studied in rat and mouse bladder carcinogenesis models and examined clinically as a chemopreventive agent in bladder.[61,62] However, in Phase III prevention trial of fenretinide in patients with resected NMIBC, it was suggested that fenretinide was ineffectual to decrease the recurrence rates in patients with Ta, T1 or Tis stages of bladder cancer.[62] Nevertheless, subgroup analysis demonstrated that high-risk patients receiving both Bacillus Calmette Guerin (BCG) and 4-HPR treatment were presumably 1/3 less likely to develop recurrence in comparison to placebo. The authors have asserted that BCG treatment could promote the activity of fenretinide. In another study, Decensi et al suggested that fenretinide was ineffectual in regulating the flow cytometric DNA content distribution and the morphology of urothelial cells.[61] Since these observations are obstructed by the lack of predictivity of the reliable surrogate endpoint biomarkers, further studies are required to define the role of fenretinide in treatment of bladder cancer.

Treatment with another synthetic retinoid, Etretinate (Tegison) was shown to decrease the annual transurethral resection rate significantly in patients with superficial papillary bladder tumors.[63,64] However, Etretinate was shown to result in birth defects. In another study, treatment with combined ketoconazole (a strong inhibitor of RA-catabolizing cytochrome P450s) and ATRA (Keto-ATRA) therapy

TABLE 1 Applications of retinoic acid and retinoid derivatives in bladder cancer

Name of the chemical	Application	Molecular and phenotypic effects
13-*cis*-retinoic acid	Normal human urothelial cells	The inhibition of squamous metaplasia and reverting to basal phenotype[54]
Phenylretinamides (including 4-HPR)	Transitional cell carcinoma of the bladder cell lines	The inhibition of cell growth and the induction of apoptosis[55]
4-HPR	Human bladder cancer cell lines	The induction of apoptosis and G1 cell cycle arrest, and the inhibition of cell growth[56]
ATRA	Bladder cancer cell lines	Dose-dependent reduction in cell growth[57]
13-*cis*-retinoic acid	Rat bladder carcinogenesis model	The inhibition of the incidence and the severity of neoplastic urothelial alterations[58]
13-*cis*-retinoic acid, N-(ethyl)-all-*trans*-retinamide or N-(2-hydroxyethyl)-all-*trans*-retinamide	Rat urinary bladder carcinogenesis model	Reducing the number and severity of the transitional cell carcinomas[59,60]
4-HPR	Patients with resected superficial bladder cancer	A lack of effect on the DNA content distribution and the morphology of urothelial cells and lack of activity on tumor recurrence[61]
4-HPR	Patients with transitional cell carcinoma of the bladder	No significant effect to reduce the recurrence rates[62]
Etretinate	Patients with superficial bladder tumors	The decrease of the annual transurethral resection rate[63,64]
Ketoconazole + ATRA	Superficial bladder cancer patients	The improvement of survival time and decrease in recurrence rate[65]
4-HPR	Patients with superficial bladder cancer	Reduction of IGF-I levels[66]
Vitamin A	Patients with transitional cell carcinoma of the bladder	A decrease in bladder tumor recurrence in patients receiving BCG immunotherapy[67]

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influentially decreased recurrence rate and enhanced survival time of superficial bladder cancer patients, presumably via decrease in the activity of the primary mitogenic/angiogenic factors such as VEGF and TGFα; likely by averting the malignant cells.[65] In another study, 4-HPR was shown to modulate circulating insulin-like growth factor (IGF)-I, IGF-II and Insulin-like growth factor-binding protein (IGFBP)-3 levels in patients with superficial bladder cancer.[66] Furthermore, Vitamin A was shown to result in a decrease in bladder tumor recurrence in patients receiving BCG immunotherapy.[67] In addition, retrospective population studies have proposed a decreased incidence of bladder carcinoma for those taking a high dietary levels of vitamin A. Some published literature from clinical trials advocates the usage of vitamin A for the secondary prevention of bladder cancer.[68] Despite this evidence, further investigation is necessary to adequately define the specific role of retinoids in the treatment of bladder cancer.

6 | RA SIGNALING AND EPIGENETIC REGULATION

The abnormal epigenetic landscape is a hallmark of cancer. An altered pattern of epigenetic modifications contributes to tumorigenesis by causing transcriptional deregulation ultimately dysregulating basic cellular processes resulting in aberrant proliferation, impaired differentiation and impaired apoptosis.[69]

Dysregulation of the epigenome through defects in DNA methylation, histone modifications, chromatin remodeling and the regulation of noncoding RNA affects the structure and integrity of the genome and disrupts normal patterns of gene expression. Typically, the transcriptional silencing of tumor-suppressor genes by CpG-island-promoter hypermethylation is one of the most known epigenetic dysregulation mechanisms, contributing to all common hallmarks of cancer.[70,71]

Preternatural enriched mutational spectrum of epigenetic machinery has been observed in bladder cancer, affecting DNA methylation,[72] chromatin organization,[7] histone modifications[8] and the expression of noncoding RNAs.[7,8,73] Remarkably, almost 80% of bladder cancer patients are mutant for at least one gene involved in the shaping of epigenetic landscape (Figure 1).[8] This situation strongly argues that epigenetic machinery may be a putative target for bladder cancer management, a source of useful biomarkers for diagnostic, prognostic and prediction of response, as well as a novel area of research with incredible new insights into the molecular mechanisms of bladder cancer biology.

Beyond the well-established effects of RA signaling on specification of cell lineage via transcriptional activation of RAR-inducible genes, recent studies revealed that RA further modulates cell differentiation through rapid and broad effects on the epigenome. This discovery paves the way for future clinical applications of RA, a new area of fundamental research in transcriptional control.

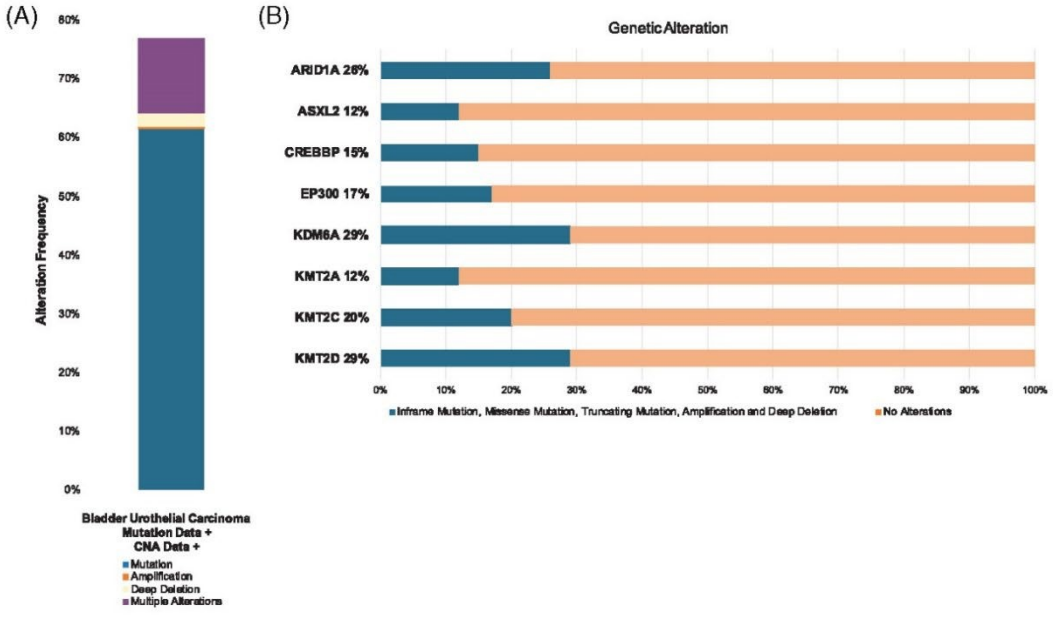


FIGURE 1 Mutation frequency of chromatin modifiers in bladder cancer. The figure was generated using the mutation data generated in muscle-invasive bladder cancer[8] and the cBioPortal.[74] A, The cumulative alteration frequency of the chromatin modifier genes, *ARID1A*, *ASXL2*, *CREBBP*, *EP300*, *KDM6A*, *KMT2A*, *KMT2C* and *KMT2D*. B, Individual mutation frequency of the chromatin modifiers in (A)

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RA affects the shaping of the epigenome in multiple ways (Figure 2). The reciprocal existence of active H3K4me3 signals and repressive H3K27me3 signals is denominated as bivalent domain. The genes which are marked by bivalent chromatin configuration have balanced levels of H3K4me3 and H3K27me3 and the relative changes in the levels of these histone modifications are coupled with transcriptional activation or repression. Bivalent chromatin structure frequently exists within the promoters of RA inducible genes.[16,17,75-78] The contrariwise activities of H3K4me3 and H3K27me3 are corroborated by the observation that RA-induced transcription caused to a simultaneous decrease in H3K27me3 levels as well as an increase in H3K4me3 levels, for instance, this has been shown for the Hoxa cluster.[16]

Curiously, by containing Lysine demethylases (KDM) 6A (KDM6A = UTX), an H3K27 demethylase, MLL2 complex integrates methyltransferase action targeting H3K4 with demethylase action targeting the contrary H3K27me3 mark.[79] Urvalek et al refer to this situation as "push-pull effect".[18] A similar "push-pull" activity is conferred to The Polycomb repressive complex 2 (PRC2) that involves EZH2, an H3K27 methyltransferase, and KDM5B, an H3K4 demethylase. This push-pull effect and resolution of bivalent chromatin domains upon RA induction have been shown in several studies.[18]

Another aspect, which links RA-signaling with bivalent domains, is the regulation of DNA methylation. It is known that thymine DNA glycosylase (TDG), a base excision repair enzyme, maintains bivalent chromatin domains in embryonic stem cells by facilitating base excision repair with the purpose of antagonizing aberrant DNA methylation.[80] Given the fact that numerous RA major target genes are located in bivalent domains, Um et al established connections between TDG and the RARs/RXRs that may connect RA to active demethylation of DNA.[81] It was also identified that TDG action was a pre-requisite for the recruitment of the coactivator protein KAT3B (p300) to the promoters of RA-target genes.[18,82] Therefore, a defect in TDG function may eventuate with a reduction in RAR/RA-related gene transcription and defects in cell differentiation.[83]

In addition to the changes in the levels of bivalent histone marks upon RA stimulation as explained above, H3K27, H3K9 and H3K14 are acetylated with RA induced transcriptional activation.[16,76] The RA-dependent recruitment of the acetyltransferases KAT3B (p300) and KAT13B (NCoA3, pCIP, Src3) to the RAREs of certain target genes indicate that these KATs also serve vital functions in RA-induced transcription.[4,14]

7 | COMBINATORIAL RA AND EPI-DRUG TREATMENT OPTIONS IN BLADDER CANCER

The utilization of RA in combination with epigenome-modifying medicines is promising for treating and/or preventing of many forms of cancer. These kinds of combination therapies are becoming increasingly favorable, as various types of cancers show aberrant mutational load in epigenetic regulatory proteins.

Recently, in several clinical trials, ATRA has been used as part of combination therapies with other epigenetic drugs (Figure 3, Table 2)

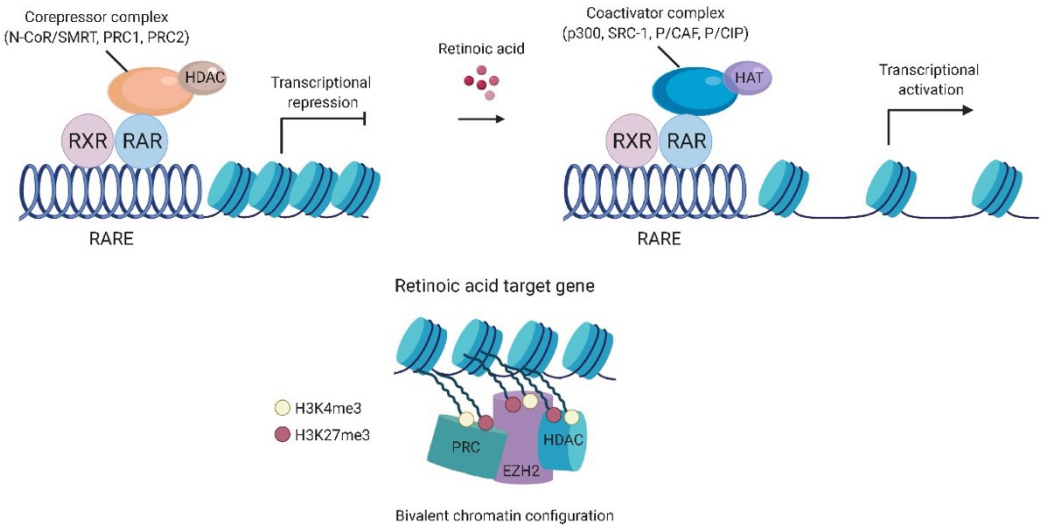


FIGURE 2 RA signaling and epigenetic regulation. RA regulates gene expression through broad effects on the epigenome. The simultaneous existence of active H3K4me3 signals and repressive H3K27me3 signals represents "bivalent domain". By means of the relative changes in the levels of these histone modifications, gene transcription is activated or repressed. By virtue of numerous RA target genes exhibit bivalent signatures, the transcription of RA target genes are closely associated with the concept of bivalent marks

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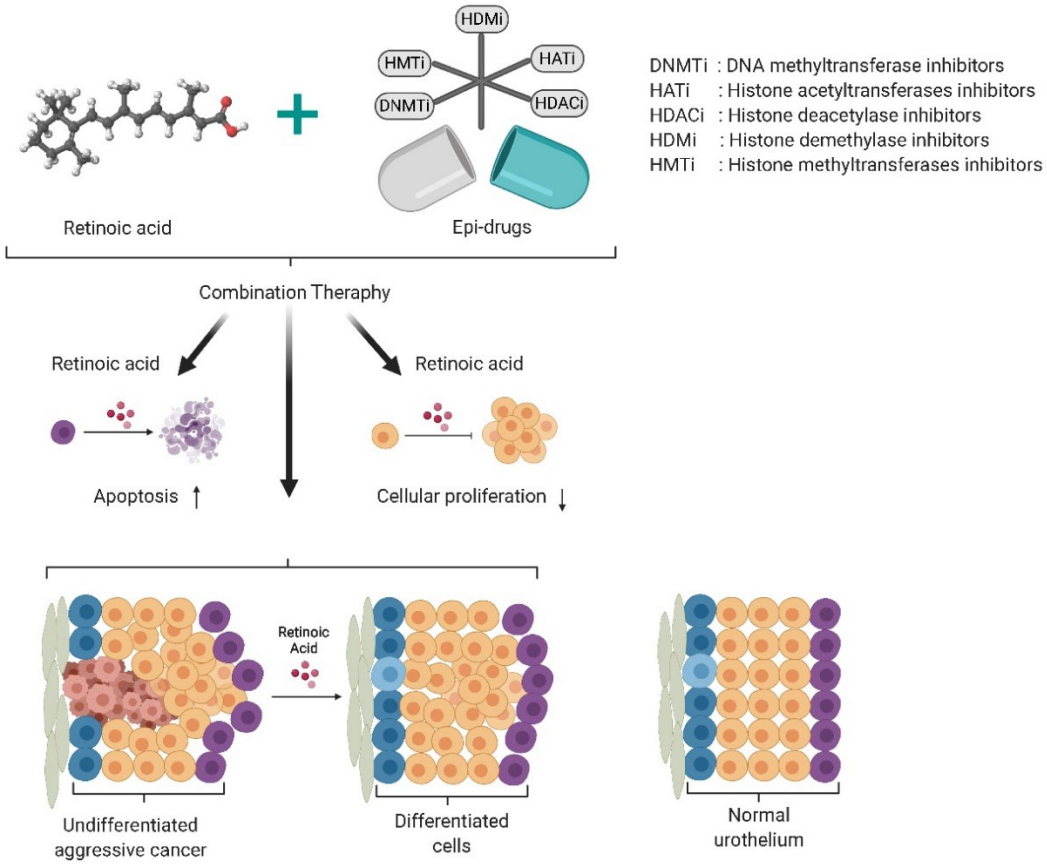


FIGURE 3 Co-targeting RA signaling and epigenetic machinery in bladder cancer. The combinations of retinoids and epigenetic modulating drugs could be rewarding treatment options for bladder cancer, mainly due to their actions to facilitate cell differentiation, to promote apoptosis and to inhibit cell proliferation

in a variety of cancers including lung, prostate, breast, ovarian, liver and bladder cancer.[13,62,84] It is thought that such combinatorial applications could provide a basis for the elimination of repressive epigenetic marks, thereby re-sensitizing cells to growth and differentiation signals in cancer.

In the case of using ATRA combined with histone deacetylase inhibitors (HDACi) (such as TSA and valproic acid), RA has been shown to re-stimulate RARβ expression in cervical cancers and renal cell carcinoma[88,89], obstruct cell proliferation in various kinds of malignancies such as renal cell carcinoma, AML, melanoma and breast cancer[84,85,90-93] and induce differentiation and result in a better clinical response in APL.[86,87] HDACi which are in clinical trials at different phases[100,101] exist for various types of cancers including bladder cancer. The combination of 13-*cis*-RA and HDACi entinostat was demonstrated to induce histone acetylation in patients with solid tumors including urothelial carcinoma.[84] Similarly, in another study, combination of 13-*cis*-RA and vorinostat had resulted in increased histone acetylation in neuroblastoma patients.[99]This combination strategy was based on previous preclinical models suggesting that histone deacetylase inhibitors could enhance sensitivity of epithelial tumors to retinoids.[89,91,93,94,102]

Alternative promising treatment method is the co-administration of retinoids in conjunction with DNA methyltransferase inhibitors. Combination therapy of RA and the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-Aza) showed increased antineoplastic effects in head and neck cancer and oral squamous cell carcinoma[95,96] as well as yielded promising results in AML patients in a phase II clinical trial.[97] In the case of non-APL AML, epi-drugs such as the ones

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TABLE 2 Combinatorial applications of retinoic acid and epi-drugs

Name of the chemicals (RA + epi-drug)	Application	Molecular and phenotypic effects
Entinostat + 13-*cis*-retinoic acid	Patients with solid tumors including urothelial carcinoma, prostate, kidney and breast cancer	Induction of histone acetylation and partial clinical benefit[84]
RA + 5-aza-2′-deoxycytidine + valproic acid	Breast cancer	The inhibition of proliferation[85]
ATRA + HDACi sodium phenylbutyrate	APL	Induction of differentiation[86] and impressive control of the disease[87]
ATRA + valproic acid	Cervical cancer	Restored RARβ2 expression and induced differentiation[88]
ATRA + HDACi trichostatin	Human renal cell carcinoma	An additive inhibition of cell proliferation[89]
ATRA + valproic acid	AML	Reprogramming differentiation[90]
13-*cis*-retinoic acid + HDACi LAQ824	Human malignant melanoma	Increased antitumor activity[91]
13-*cis*-retinoic acid + SAHA	Human renal cell carcinoma lines	Induced several transcripts with known anticancer/immunomodulatory effect[92]
13-*cis*-retinoic acid + HDACi MS-275	Human renal cell carcinoma	Reverting retinoid resistance by epigenetic silencing of RARβ2[93]
13-*cis*-retinoic acid + phenylbutyrate	Prostate cancer	Additive inhibitory effects on tumor growth[94]
RA + 5-Aza + valproic acid	Head and neck cancer	Enhanced growth inhibition in cells resistant to VPA[95]
RA + 5-Aza	Oral squamous cell carcinoma	Most effective to reduce oral cavity tumorigenic progression[96]
RA + 5-Aza	AML	Not highly effective in AML except in the presence of the PML-RARA fusion protein[97]
ATRA + TCP	AML	A superior potent antileukemic effect[98]
13-*cis*-retinoic acid + vorinostat	Neuroblastoma patients	Increased histone acetylation[99]

against lysine-specific demethylase 1 (LSD1), which is a H3K4 demethylase, are most effective in conjunction with ATRA.[98]

Remarkably, almost all the chromatin modifiers that are mutated in bladder cancer are involved in active chromatin organization. Among those KDM6A, which functions in MLL complex, antagonizing polycomb mediated repression is mutated with 26% frequency in bladder cancer.[8] In this respect, inhibitors against EZH2, one core component of the polycomb repressive complex, have been shown to reduce bladder cancer growth and metastasis in bladder cancer cell lines.[103] Furthermore, there is a phase I/II clinical study for testing the effects of tazemetostat, EZH2 inhibitor, in treating patients with advanced and metastatic bladder cancer.[104] Therefore, the combinational use of EZH2 inhibitors with RA-analogues in bladder cancer patients could be a viable therapeutic strategy.

In conclusion, some clinical trials offer avenues for potential rational therapeutic combinations of retinoids with HDAC inhibitors or EZH2 inhibitors in bladder carcinomas. However, toxicity remains an obstacle and further consideration of lower dose regimens could be required. To restore the therapeutic effects of RA, many studies emphasize the need for a better understanding of aberrant epigenetic mechanisms that block RA signaling and negatively affect RA regulated gene expression in cancer.

8 | CONCLUSIONS AND PERSPECTIVES

The pleiotropic effects and the ability of retinoids to compel cells into differentiation and apoptosis offer stunning promise for cancer therapy and chemoprevention.[12] Even though pharmacological doses of retinoids have been ratified for the treatment of some hematologic malignancies and high-risk neuroblastoma, by regulatory agencies such as the Food and Drug Administration (FDA), the chemopreventive and therapeutic efficacy of retinoids in bladder cancer are inexplicit yet. Furthermore, use of epigenetic approaches in clinic remains in a nascent developmental stage. Future investigations are focusing on this emerging and highly underexplored field of combination of epi-drugs and retinoids for cancer treatment. Especially, further research is needed on the function and specificity of different lysine acetyltransferases (KATs) and KDMs corresponding with RA transcriptional activation and on how RARα (and probably other RARs) specifically play a role in the maintenance of gene expression by holding certain promoters in a hypomethylated state.[18] If validated, this could open up the possibility for retinoids to be approved and adopted into medical guidelines for bladder cancer treatment, development of epigenetic biomarkers for disease diagnosis, prognosis or response to therapy.

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It is considered that the treatment with ATRA is most efficient when combined with other drugs to target sophisticated gene silencing mechanisms and reactivate ATRA-induced gene expression and differentiation of cancer cells, as well as to modulate anticancer activities of ATRA in principle. Further explorations of such combinatory approaches may decipher multiple novel pathways/targets to reveal and vitalize the potential of ATRA cancer therapy, especially in bladder cancer. Although prospering clinical effects have been well documented, proof of retinoid-resistance in some cancers has restricted the clinical use of retinoids. Therefore, an advanced conception of RA resistance mechanisms to overcome retinoid resistance for retinoid-based treatments is warranted.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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8.2 Ethical Approval

İZMİR BİYOTIP VE GENOM MERKEZİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)
KARARI

Toplantı Tarihi	: 24/02/2023	**Toplantı Günü**	: Cuma
Toplantı Sayısı	: 3	**Toplantı Saati**	: 10:30

Sayın Doç. Dr. Şerif ŞENTÜRK,

2023-007 Protokol No'lu; sorumlusu olduğunuz "Mesane kanserinde EZH2 ve retinoik asit sinyalinin birlikte hedeflenmesi" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof. Dr. H. Alper BAĞRIYANIK **Başkan**	**Prof. Dr. Hilmi ORHAN** **Başkan Yardımcısı**
Prof. Dr. Sedef AKGÜNGÖR **Üye** (Katılmadı)	**Prof. Dr. Gülgün OKTAY** **Üye**
Dr. Serap ERKEK ÖZHAN **Üye** (Katılmadı)	**Prof. Dr. Şermin GENÇ** **Üye**
Doç. Dr. Canan Aslı YILDIRIM **Üye** (Katılmadı)	**Doç. Dr. Can KÜÇÜK** **Üye**
Dr. Öğr. Üyesi Yavuz OKTAY **Üye**	

8.3 Curriculum Vitae

GİZEM ÖZGÜN

Kişisel Bilgiler

İletişim Bilgileri

İletişim Adresi	Izmir	ı Merkezi
E-posta	gizem	r
İnternet Sayfası		

Öğrenim Bilgileri

07 Eylül 2017 - Şu Anda (6 yıl 1 ay)
Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ, MOLEKÜLER BİYOLOJİ VE GENETİK (DR) (İNGİLİZCE)
Diploma Numarası: -
Ağırlıklı Genel Not Ortalaması: 3.5 / 4.0

11 Şubat 2013 - 12 Mayıs 2016 (3 yıl 4 ay)
Yüksek Lisans, Tezli Program, EGE ÜNİVERSİTESİ, TÜRKİYE
SAĞLIK BİLİMLERİ ENSTİTÜSÜ, FARMASÖTİK TOKSİKOLOJİ (YL)
Diploma Numarası: 2015/93/0112
Ağırlıklı Genel Not Ortalaması: 3.08 / 4.0

04 Eylül 2007 - 15 Ocak 2013 (5 yıl 5 ay)
Lisans, Anadal/Normal Öğretim, EGE ÜNİVERSİTESİ, TÜRKİYE
FEN FAKÜLTESİ, KİMYA PR.
Diploma Numarası: 2012/04/0003
Ağırlıklı Genel Not Ortalaması: 2.89 / 4.0

Deneyim / İşyeri Bilgileri

01 Haziran 2011 - 01 Eylül 2011 (4 ay) (Tam Zamanlı)
STAJYER, GHENT UNIVERSITY

Yabancı Dil Bilgileri

İNGİLİZCE (Okuma: İyi, Yazma: İyi, Konuşma: İyi)

Bilimsel Teknolojik Faaliyet Alanları

Bilimsel Teknolojik Faaliyet Alanı Bilgileri

Temel Bilimler -- Yaşam Bilimleri -- Moleküler Biyoloji ve Genetik -- Kanser Moleküler Biyolojisi

Ar-Ge Yetkinlik

Makaleler

G. ÖZGÜN, Ş. ŞENTÜRK & S. ERKEK ÖZHAN, Retinoic acid signaling and bladder cancer: Epigenetic deregulation, therapy and beyond, INTERNATIONAL JOURNAL

OF CANCER, 2020, 0020-7136, 148, 10, 2364-2374.

Bildiriler

G. ÖZGÜN, T. YARAŞ, N. LANDMAN, G. KARAKÜLAH, M. V. LOHUIZEN, Ş. ŞENTÜRK & S. ERKEK ÖZHAN, A molecular portrait of retinoids and EZH2 inhibitors cooperation to orchestrate cytotoxic effects on bladder cancer cells, Poster Sunumu, 47th FEBS Congress, 08 Temmuz 2023, 12 Temmuz 2023.

G. ÖZGÜN, Ş. ŞENTÜRK & S. ERKEK ÖZHAN, Revealing the role of retinoids on the phenotypic and epigenetic landscape of bladder cancer cells, Davetli Konuşmacı, 6th International Medicine and Health Sciences Research Congress (UTSAK), 10 Nisan 2021, 11 Nisan 2021.

G. ÖZGÜN, H. UZUNER, G. KARAKÜLAH & R. L. J. MEUWİSSEN, Characterization of new biomarkers for NSCLC using the liquid biopsy technique, Davetli Konuşmacı, Onkolojide İz Bırakanlar Zirvesi, 14 Kasım 2019, 17 Kasım 2019, S203 - null.

G. ÖZGÜN, B. AĞRAP & F. LERMİOĞLU ERCİYAS, The investigation of the effects of Cinnamomum cassia extracts on Type 1 diabetes in vitro model, Poster Sunumu, 43 rd FEBS Congress, 07 Temmuz 2018, 12 Temmuz 2018.

TÜBİTAK Burs ve Destekleri

Proje Bilgileri

117S954, Kan Dolaşımındaki Tümör DNA ve RNA'sının Yüksek Hassasiyetli Analizi Yöntemini Kullanarak, Akciğer Kanseri Tanı ve Tedavi İzlemi için Yeni Biyobelirteçlerin Karakterizasyonu, Burslu, Yürürlükte, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, Uluslararası / PIA-Bosphorus-Fransa, ARDEB, Projeye Katılma/Ayrılma Tarihleri: 26.06.2019 - 15.10.2019, Proje Başlangıç/Bitiş Tarihleri: 15.06.2018 - 15.12.2022.
112S120, Cinnamomum Cassia ve C. Zeylanicum Bark Ekstrelerinin Diyabette Endojen ve H2O2 ile İndüklenen Oksidatif DNA Hasarı ve Onarımı Üzerindeki Etkilerinin In Vitro Araştırılması, Burslu, Sonuçlandı, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, 1001 - Araştırma, ARDEB, Projeye Katılma/Ayrılma Tarihleri: 01.03.2013 - Devam ediyor, Proje Başlangıç/Bitiş Tarihleri: 01.03.2013 - 01.11.2015.

BİDEB Destekleri

GİZEM ÖZGÜN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2224-A Yurt Dışı Bilimsel Etkinliklere Katılma Desteği Programı, Destekleniyor, 2023 - 2.
GİZEM ÖZGÜN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2211-C Öncelikli Alanlara Yönelik Yurt İçi Doktora Burs Programı, Başvurusu Reddedildi, 2020 - 1, 01.03.2020 - 28.02.2023.
GİZEM ÖZGÜN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2211-C Öncelikli Alanlara Yönelik Yurt İçi Doktora Burs Programı, Destekleniyor, 2020 - 2, 01.10.2020 - 30.09.2023.

Panelistlik/İzleyicilik/Raportörlük Sayısı

Hakemlik/Panelistlik/Dış Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
İzleyicilik/Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
Raportörlük Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0