Novel dual BET and PLK1 inhibitor WNY0824 exerts potent anti-tumor effects in CRPC by inhibiting transcription factor function and inducing mitotic abnormality

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Abstract

Castration-resistant prostate cancer (CRPC) is a lethal disease with few treatment alternatives once patients become resistant to second-generation anti-androgens. In CRPC, BET proteins are key regulators of AR- and MYC-mediated transcription, while the PLK1 inhibitor potentially downregulates AR and MYC besides influencing the cell cycle. Therefore, synchronous inhibition of BET and PLK1 would be a promising approach for CRPC therapy. This study developed a dual BET and PLK1 inhibitor WNY0824 with nanomolar and equipotent inhibition of BRD4 and PLK1. In vitro, WNY0824 exhibited excellent anti-proliferation activity on AR-positive CRPC cells and induced apoptosis. These activities are attributable to its disruption of the AR-transcriptional program and the inhibition of the ETS pathway. Furthermore, WNY0824 downregulated MYC and induced mitotic abnormality. In vivo, oral WNY0824 administration suppressed tumor growth in the CRPC xenograft model of enzalutamide resistance. These findings suggest that WNY0824 is a selective dual BET and PLK1 inhibitor with potent anti-CRPC oncogenic activity and provides insights into the development of other novel dual BET- and PLK1-inhibiting drugs.

Introduction

Prostate cancer (PCa) is the second most common malignancy and the fourth leading cause of death from cancer in men (1,2). For decades, the mainstay of first-line treatment for metastatic prostate cancer has been androgen deprivation therapy (ADT) (3). Despite initial responses to ADT, patients with metastatic disease invariably progress to a stage termed castration-resistant prostate cancer (CRPC) (4-6) and eventually acquire resistance to second-generation anti-androgens including enzalutamide (Enz) and abiraterone (Abi) (7,8). Multiple mechanisms potentially
result in the resistance phenotype, such as the emergence of AR ligand-binding domain (LBD) mutations and AR variants lacking the LBD (9-11). In addition, the overexpression of transcription factors, such as the ETS family, a frequent translocation target in prostate cancer, or MYC, may occur along with AR overexpression to promote resistance to hormonal therapy (12). Therefore, novel strategies to treat CRPC are urgently required.

Polo-like kinase 1 (PLK1), a serine/threonine kinase, is overexpressed in various human tumors, which is associated with a poor prognosis (13,14). PLK1 plays several key roles in mitosis, including entry into mitosis, bipolar spindle formation, chromosome segregation, and cytokinesis (15,16). In addition, it has been reported that inhibition of PLK1 triggers degradation of MYC in NB, SCLC, and DHL (17,18). Furthermore, Zhang and colleagues found that PLK1 elevation leads to constitutively active AR signaling, which sustains the development of the advanced stage of disease (19,20), implying that PLK1 inhibitors would be treatment options for AR related cancers like prostate cancer.

BRD4, a conserved member of the BET family, binds to acetylated lysine residues as well as other nuclear proteins via its tandem domains (BD1 and BD2) and subsequently assembles of transcriptional regulatory complexes containing RNA polymerase II to coordinate multiple transcriptional events (21,22). This function of BRD4 is best characterized through its role in regulating the notorious oncogene MYC (23). Dysfunction of BRD4 has been strongly associated with the development of cancer (24,25). Recently, Jonathan and colleagues found that BRD4 expression increases as patients progress from HSPC to CRPC, which is associated with poorer patient outcome (26). In addition, BRD4 can regulate AR-mediated transcription through its recruitment of AR to their downstream genes in CRPC, which is crucial for prostate cancer proliferation (27). Therefore, BRD4 may be a promising therapeutic target for CRPC therapeutics.

Considering that both PLK1 and BRD4 regulate AR pathway and MYC level, which are critical to the development of CRPC, the dual inhibition of PLK1 and BET might provide a new strategy to treat CRPC. A recent report has demonstrated that a combination treatment of PLK1 inhibitor GSK461364A and BRD4 inhibitor JQ1 has a strong synergistic effect on CRPC progression (28). However, drug combination always brings drug-drug interactions and side effects. A dual inhibitor could exert similar favorable effect as drug combination do while avoid their safety risk to a certain extent and reduce the cost of drug development for combinations (29). Thus, we aimed to develop dual PLK1/BET inhibitors as anti-CRPC drugs.

Knapp and Schönbrunn independently reported that the PLK1 inhibitor BI-2536 is also a potent inhibitor of BET (30,31), however, its binding affinity to BRD4 ($K_i = 56$ nM) is 250-fold weaker than that of PLK1 ($K_i = 0.22$ nM) (32). We designed and synthesized numerous dual BET and PLK1 inhibitors. Among these candidates, WNY0824 effectively and equipotently inhibits BRD4 and PLK1. Herein, the anti-CRPC activity and molecular mechanism of WNY0824 were evaluated both in vitro and in vivo.

**Materials and Methods**
Kinase assay

For IC_{50} values, WNY0824 was tested at various concentrations ranging from 0.04 to 10 μM using radiometric ATP-competitive kinase assay (Eurofins Millipore KinaseProfiler™ Service Assay). The broader kinase selectivity profile of WNY0824 was assessed against a panel of 418 kinases using the Eurofins Millipore KinaseProfiler™ Service.

Cellular Thermal Shift Assay

After treatment with WNY0824 or DMSO for 2 h, cells were harvested, washed by PBS, and then lysed in RIPA buffer with protease inhibitors and phosphatase inhibitors. The lysates were centrifuged with 15000 × g for 20 minutes at 4°C. The soluble fractions were divided into smaller (25μL) aliquots, heated at different temperatures for 5 minutes, and centrifuged with 15000 × g for 20 minutes at 4°C. Then the soluble fractions were transferred to new microtubes and analyzed by western blotting. The antibodies used in this article are BRD4 (CST, 13440) and PLK1 (Abways, CY5844).

Cell lines and Cell culture

Human prostate cancer cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) in 2017. LNCaP, 22RV1, DU145 and PC3 cells were cultured in RPMI 1640 (Gibco); VCaP cells were cultured in DMEM (Gibco); all were supplemented with 10% FBS and 1% antibiotics (Penicillin-Streptomycin) and then incubated in 5% CO_{2} cell culture incubator. All cell lines were characterized by PCR and STR analysis for contamination and authentication.

Cell viability assay

Cells were seeded in 96-well plates at 2~10 ×10^{3} cells per well with a total volume of 100 μL media and incubated for 24 h. Then different doses of compounds in 100 μL media were added to the cells. After indicated times, cell viability was determined using an MTT (Sigma-Aldrich) assay. For colony formation assay, 10,00–5,000 cells per well were seeded in 6-well plates and treated with WNY0824 or DMSO. After 12 days, cells were fixed with 4% formaldehyde, stained with 0.05% crystal violet and photographed.

Cell cycle and apoptosis analysis

Cells were seeded in 6-well plates and treated with WNY0824 or DMSO for indicated times. For cell cycle analysis, cells were washed with PBS, fixed in 70% ethanol overnight, stained with propidium iodide for 10 min in dark and analysed by flow cytometry. For apoptosis analysis, cells were harvested, washed with PBS, stained with Annexin V-FITC apoptosis detection kit (Roche) for 5 min in dark and analysed by flow cytometry.

RNA isolation and quantitative real-time PCR (qRT-PCR)
Total cellular RNAs were extracted and cDNA was synthesized using the first strand cDNA synthesis Kit (Mei5). The qRT-PCR reactions were carried out using the SYBR Green RT-PCR Kit (Takara) according to the manufacturer’s instructions. The primer sequences used in this article were listed in the Supplementary Tab. S1.

**ChIP and ChIP-qPCR**

VCaP prostate cancer cells were treated as described. The ChIP assays for BRD4, AR were performed using the Magna ChIP A/G Chromatin Immunoprecipitation Kit (Merck Millipore) according to the manufacturer’s instructions. The antibodies used for ChIP assay are AR (CST catalogue no. 5153S) and BRD4 (CST catalogue no. 13440S). qPCR was performed with SYBR (Takara), using specific primers (listed in Supplementary Tab. S2) on a Bio-Rad CFX96, and analyzed using ΔΔCt method.

**Western blot (WB), Immunofluorescence (IF), and immunohistochemistry (IHC) analyses**

The WB, IF, and IHC analyses were performed according to standard methods. The antibodies used in this article were listed in the Supplementary Tab. S3.

**Pharmacokinetic analysis**

All animal experiments have been approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number: 20170135). Sprague-Dawley rats were fasted overnight before dosing. WNY0824 was administered at 3 mg/kg or 30 mg/kg in 5% DMSO, 30% PEG400 and 65% saline solution 25%. Blood was collected into EDTA-containing tubes for plasma at different times. The plasma samples were isolated by centrifugation, and the concentrations of WNY0824 were determined using LC-MS/MS.

**Xenograft mouse model**

5×10⁶ 22RV1 cancer cells were suspended in 100 μL of serum-free medium with 50% Matrigel (BD Biosciences) and injected subcutaneously into the NOD-SCID mice (5–6 weeks). Once the tumors had grown to 100 mm³, the animals were randomized into 4 groups and treated with vehicle, Enzalutemide (10 mg/kg), or WNY0824 (30 or 60 mg/kg) via oral gavage once a day for 18 days. The tumors were measured by a vernier caliper every three days, and tumor volumes were calculated as follows: V = L× W²/2 (V: volume, mm³; L: length, mm; W: width, mm). Mice were killed and tumors were extracted, weighed, and fixed in formalin at the end of this study.

**Results**

**Biochemical characterization of WNY0824**

We designed and synthesized numerous dual BET and PLK1 inhibitors. Among these candidates, WNY0824, which was characterized by (R)-4-((5-(cyclohexylmethyl)-4-ethyl-1-methyl-4,5-dihydro-[1,2,4]triazolo[4,3-f]pterid...
(din-7-yl)amino)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamide (Fig. 1A), was selected as the primary compound for its preferable and equipotent effects on BET and PLK1. The preparation of WNY0824 was shown in Supplementary methods.

In biochemical assays, WNY0824 displayed potent and concentration-dependent inhibition of BRD2, BRD3, BRD4, and BRDT with half-maximal inhibitory concentration (IC$_{50}$) values of 402.5 ± 3.6 nM, 150.7 ± 4.2 nM, 109.3 ± 5.1 nM, and 311.9 ± 2.8 nM, respectively (Fig. 1B). Furthermore, WNY0824 inhibited PLK1 kinase activity in vitro, with an IC$_{50}$ of 22 nM, as determined via a radiometric ATP-competitive kinase assay (Fig. 1C). Its equipotent inhibition of BRD4 and PLK1 ensured that the activity of both targets could be simultaneously restrained, which provide us a valuable probe to explore the advantages of dual BET and PLK1 inhibitors in cancer therapy.

To assess the potential of WNY0824 for inhibiting off-target kinases, its activity against 418 human kinases and their relevant mutants was evaluated at 1 μM (Fig. 1D, Supplementary Tab. S4). The strongest target inhibition was observed with PLK1. Moreover, the inhibition ratio of WNY0824 against almost 96% of the kinases tested was <35%, confirming the excellent targets selectivity of WNY0824.

In addition, cellular thermal shift assay (CETSA) showed that WNY0824 increased the thermal stability of PLK1 and the temperature shift heightens with the increase in concentration of WNY0824 (Fig. 1E). Furthermore, the thermal stability of BRD4 was enhanced upon treatment with 10 μM WNY0824 although the concentration-dependent increase of thermal stability was not observed (Fig. 1F). Overall, these data indicated that WNY0824 was a selective dual inhibitor of BET and PLK1.

**Efficacies of WNY0824 against human prostate cancer cell lines**

We screened a panel of 5 human PCa cell lines (LNCaP, 22RV1, VCaP, PC3, and DU145), wherein PLK1 and BET were ubiquitously expressed (Fig. 2A), to assess their sensitivity to WNY0824 in a 4-d growth assay. The dose-response curves of WNY0824 and IC$_{50}$ values were shown in Fig. 2B and 2C. Although growth inhibition was observed to a certain extent in all cell lines, the AR-positive cells (LNCaP, 22RV1, and VCaP) were preferentially sensitive to WNY0824, with IC$_{50}$ values ranging from 110 nM to 280 nM. In addition, proliferation curve of PCa cell lines showed that the anti-proliferation activity of WNY0824 against PCa cells was in a dose-dependent and time-dependent manner (Fig. 2D and Supplementary Fig. S1A). Furthermore, colony formation assay (Supplementary Fig. S1B) showed that WNY0824 reduced the number and size of colonies in 22RV1 and LNCaP cells more notably at all concentrations, as compared with that for AR-negative DU145 and PC3 cells.

We then assessed apoptotic induction upon treating PCa cells with WNY0824. FCM analysis revealed that exposure of LNCaP, VCaP, and 22RV1 cells to WNY0824 for 48h significantly increased the population of apoptotic cells (Annexin V-positive) in a concentration-dependent manner (Fig. 2E). Apoptosis related morphological changes, including cell shrinkage, nuclear fragmentation, and
chromatin condensation with brighter-blue fluorescence were also enriched in LNCaP and 22RV1 cells treated with WNY0824 (Supplementary Fig. S2A). In addition, cleaved caspase-3 and cleaved PARP were upregulated with an increase in the WNY0824 concentration, while Bcl-2 was downregulated (Fig. 2F). Previous study demonstrated that BRD4 inhibitors upregulated the anti-apoptosis protein Mcl-1 through repression of Mcl-1-targeting microRNAs, which might impair their anti-proliferative activity in acute myeloid leukemia (33). PLK1 inhibition directly destructed Mcl-1 through enhancing Fbw7 activity in DHL (18). In LNCaP, 22RV1, and VCaP cells, Mcl-1 was slightly upregulated upon JQ1 treatment, resembling that in AML, while the expression of Mcl-1 with BI-2536 and WNY0824 exposure were remarkably suppressed, indicating that the BRD4-inhibition-induced Mcl-1 upregulation of WNY0824 might be compensated by its upregulation effect of PLK1 inhibitory activity (Fig. 2F). We also observed the population of apoptotic cells, cleaved caspase-3 and cleaved PARP were upregulated, although to a less extent, in AR-negative DU145 and PC3 cells with WNY0824 exposure (Supplementary Fig. S2B and S2C).

Together, these data indicate that WNY0824 potentially inhibits proliferation and induces apoptosis in LNCaP, 22RV1, and VCaP cells in a time-dependent and concentration-dependent manner. We then investigated the function mechanism of WNY0824.

WNY0824 repressed AR-regulated pathway

Disruption of the AR-transcriptional program is a promising method for CRPC treatment (34,35). PLK1 elevation leads to increased levels of AR (20), and BRD4 essentially regulates AR-mediated transcriptional activity through direct interaction with AR in the nucleus and their recruitment to AR-regulated genes (27). Thus, we speculated that our dual PLK1/BET inhibitor WNY0824 could impair AR-transcriptional program by downregulating of AR expression and inhibiting the interaction of BRD4 and AR in CRPC cell lines, simultaneously. The AR-regulated pathway in 22RV1, VCaP, and LNCaP cells were then evaluated.

Western blot and qRT-PCR analysis showed that AR mRNA level and AR protein level was almost unaffected when treated with a therapeutically relevant dose of JQ1, while BI-2536 led to a reduction of both AR mRNA and AR protein (Fig. 3A, 3B and Supplementary Fig. S3A, S3B). Noticeably, the levels of AR mRNA and AR protein in WNY0824-treated groups were also decreased in a concentration dependent manner, suggesting that WNY0824 suppressed AR expression through inhibiting PLK1.

Next, we evaluated the effects of WNY0824 on BRD4 and AR occupancy at the PSA and TMPRSS2 enhancer via chromatin immunoprecipitation. As shown in Fig. 3C, WNY0824 caused detrimental effects on AR and BRD4 occupancy at the PSA and TMPRSS2 enhancer in VCaP cells. In addition, DHT stimulation of VCaP cells for 12 h increased the interaction of AR and BRD4 at the androgen response element of the PSA and TMPRSS2 enhancer, which was inhibited upon WNY0824 pretreatment.
Furthermore, the AR-regulated gene PSA (also known as KLK3) was significantly transcriptionally suppressed upon WNY0824 treatment (Fig. 3D), resulting in dose-dependent reductions in protein level (Fig. 3E). In addition, ERG, ETV1, TMPRSS2, SLC45A3, FKBP5, and BMPR1B mRNAs, regulated by AR, were downregulated to a certain extent upon WNY0824 treatment in 22RV1, VCaP, and LNCaP cells (Fig. 3F), but not in AR-negative DU145 and PC3 cells (Supplementary Fig. S3C), suggesting that WNY0824 could inhibit the expression of AR target genes effectively in an AR-dependent manner.

WNY0824 repressed the ETS pathway and downregulated MYC level

The ERG gene and the related ETS family member ETV1 are frequent translocation targets in prostate cancer and their associated overexpression augments invasion and metastasis (36,37). We investigate the effects of JQ1, BI-2536, and WNY0824 on the ETS target gene CRISP3 in 22RV1, VCaP, and LNCaP cells. BI-2536 had minimal influence on CRISP3 transcription but that of JQ1-treated groups and WNY0824-treated groups was significantly lower than the control groups (Fig. 4A), indicating that WNY0824 could suppress the ETS pathway through its inhibition of BRD4.

BRD4 regulates MYC transcription in a number of tumors by its recruitment to the super-enhancer of MYC (23,38), and PLK1 counteracts Fbw7-mediated MYC degradation (17). The dual PLK1/BET inhibitor WNY0824 was thus thought to downregulate the MYC level by inhibiting the transcription as well as accelerate the degradation of MYC. Hence, we investigated the effects of JQ1, BI-2536, and WNY0824 on MYC level in 22RV1, VCaP, and LNCaP cells via qRT-PCR and immunoblot analyses. As shown in Fig. 4B and 4C, Exposure of BI-2536 resulted in minimal loss in MYC mRNA levels but substantial decrease in MYC protein, possibly due to the upregulation of Fbw7 protein. Treatment with JQ1 also led to significant decrease in MYC protein without observable change in Fbw7 protein level, primarily attributing to its downregulation of MYC mRNA. Moreover, conspicuous dose-dependent reductions of MYC mRNA and protein and Fbw7 upregulation were observed upon exposure of WNY0824 in AR-positive cells. Taken together, WNY0824 not only suppressed MYC expression but also induced the degradation of MYC in AR-positive cells.

It has been reported that BET is not required for transcription of MYC in PC3 and DU145 cell lines (39). Thus, we next sought to determine if WNY0824 downregulated MYC level in DU145 and PC3. As shown in Supplementary Fig. S4A and S4B, JQ1 failed to reduce levels of MYC mRNA or MYC protein in either cell line. BI-2536 and WNY0824 had no effect on MYC transcription but also resulted in the downregulation of MYC protein to a lesser extent, mainly through Fbw7-mediated degradation, which could partly explain their anti-proliferative activity in these cell lines.

WNY0824 disrupted spindle formation and induced cell accumulation in G2/M phase

PLK1 and BRD4 are both intricately involved in cell cycle (16,40). We analyzed
the distribution of cell cycle phases in PCa cells via FCM analysis after treatment with JQ1, BI-2536, or WNY0824 for 24 h. As shown in Fig. 5A and Supplementary Fig. S5A, a substantial accumulation of all five cells in the G2/M phase was detected after exposure to BI-2536, in accordance with its effect in other cancer cell lines. JQ1 induced cell cycle arrest at the G0/G1 phase in 22RV1, LNCaP, and VCaP cells but had no effect on cell cycle in DU145 and PC3 cells. The dual-PLK1/BET inhibitor WNY0824 caused a significant increase in the cell population in the G2/M phase of the cell cycle in a concentration-dependent manner in all five cells, suggesting that WNY0824 was more akin to a PLK1 inhibitor with respect to its effect on cell cycle. In addition, we observed that after being exposed to WNY0824, LNCaP cells, which were synchronized at the G1/S boundary, progressed through the S phase normally at 13 h but were then arrested at the G2/M phase (Supplementary Fig. S5C, S5D).

Progression of cell cycle is regulated by various cyclins and related cyclin-dependent kinases (CDKs). Therefore, we analyzed the expression of CDKs and cyclins in LNCaP, 22RV1, VCaP, and DU145 cells. As shown in Fig. 5B and Supplementary Fig. S5B, JQ1 had no effect on cell cycle-related proteins in DU145 cells but inactivated all CDKs and cyclins in AR-positive cells, indicating that JQ1 only prevents AR-positive cells from entering the cell cycle but did not affect cell cycle in DU145 cells. While treatment with BI-2536 and WNY0824 for 24 h resulted in downregulation of cyclin D1, CDK4, cyclin E, and CDK2 protein as well as upregulation of cyclin B1, CDK1, and phospho-H3 (p-H3) in all four cell lines, suggesting that WNY0824 could induce mitotic arrest possibly by PLK1 inhibition. Furthermore, WNY0824-treated LNCaP cells showed a significant delay in mitotic entry (Supplementary Fig. S5E), which was in accordance with the effect of BI-2536 on mitosis (41).

PLK1 play crucial roles in bipolar spindle formation and accurate segregation of chromosomes during cell division (42). In order to assess whether the prolonged mitotic arrest induced by WNY0824 was consistent with its inhibition of PLK1 function, we determined the effect of WNY0824 on mitotic spindles. IF imaging showed that WNY0824-treated 22RV1 and VCaP cells showed monopolar or multipolar spindles formation (Fig. 5C), consistent with the phenotype upon PLK1 interference or PLK1 inhibitor treatment (43,44). Moreover, WNY0824-treated LNCaP cells exhibited abnormalities in spindle formation, resulting in the failure of sister chromatids to separate normally (Supplementary Fig. S5F). This result may partly explain the G2/M arrest and apoptosis induction of WNY0824 that it could prevent the normal organization of mitotic microtubules, ultimately leading to cell death.

In vivo efficacy of WNY0824

In vivo pharmacokinetic (PK) profiles were further evaluated for WNY0824. SD rats orally administering 30 mg/kg of WNY0824 resulted in a maximum concentration (Cmax) of 337.32 μg/L (588 nM, 5-fold of IC50 for BRD4 and 26-fold of IC50 for PLK1) with time of maximum concentration observed (Tmax), elimination half-life (t1/2), and AUC0-24h of 4.00 h, 4.12 h, and 1223.07 (μg/L)·h (Supplementary
Tab. S5), and the oral bioavailability of WNY0824 (30 mg/kg) was 17.27% compared to that intravenous injection. Oral dosages of 30 and 60 mg/kg were thus selected for further in vivo pharmacodynamics studies.

In a 22RV1 mouse xenograft model. Tumor-bearing mice were treated daily with WNY0824 at 30 and 60 mg/kg, with enzalutamide at 10 mg/kg, or with the vehicle through oral administration. WNY0824 displayed antitumor activities in a dose-dependent manner during the 18-day treatment period with 54% tumor growth inhibition (TGI) at 60 mg/kg (Fig. 6A, 6B), whereas enzalutamide exerted a less pronounced effect. Otherwise, WNY0824 was well tolerated during the treatment period, as apparent from the body weight of the animals and their general behavior (Fig. 6C). Furthermore, no significant difference was observed upon hematological analyses and HE staining of the primary organs between the WNY0824-treated and the vehicle-treated groups (Supplementary Fig. S6C, S6D).

To further investigate the mechanisms underlying the antitumor effects of WNY0824 in vivo, immunoblot analyses, immunohistochemical analysis, and qRT-PCR analysis were performed using tumor tissues resected from 22RV1 models on day 18. The WNY0824 group displayed a certain reduction of MYC, PSA, TMPRSS2, and BMPR1B mRNA as well as MYC protein than the vehicle-treated group (Fig. 6D, 6E). Upregulated CDK1 and cyclin B1 were also observed in the WNY0824 group (Fig. 6E), implying G2/M phase arrest after drug exposure. Furthermore, an increase in cleaved caspase-3 and a reduction in Ki-67 upon WNY0824 treatment in IHC analysis indicated the apoptosis induction effect of WNY0824, which was further confirmed by the upregulation of cleaved PARP (Fig. 6E, 6F and Supplementary Fig. S6A, S6B). Overall, these findings indicate that WNY0824 could decrease the expression of AR-BRD4-regulated genes and induce cell cycle arrest and apoptosis to exert its antitumor activity in human CRPC xenograft models by inhibiting both PLK1 and BRD4 simultaneously.

**Discussion**

It is well established that AR and MYC are essential gene for promoting invasion or resistance to hormonal therapy in AR-positive CRPC and both PLK1 and BRD4 regulate AR and MYC. Several studies have proved that BRD4 inhibitors and PLK1 inhibitors can inhibit tumor growth in CRPC xenograft mouse models by regulate the function of AR and MYC (20,27,45). And the combination study of PLK1 inhibitor GSK461364A and BRD4 inhibitor JQ1 reported recently further demonstrated the advantages of targeting at BRD4 and PLK1 in CRPC simultaneously (28). Recently, several dual BRD4/PLK1 inhibitors with similar potency against BRD4 and PLK1 have been disclosed and they displayed potent anti-proliferation activity on AML cells (32,46), while their anti-tumor activity against CRPC has not been investigated. For the first time, this study reported the anti-tumor effect of a dual BET and PLK1 inhibitor, WNY0824, in AR-positive CRPC, implying that dual BET and PLK1 inhibitors might provide an alternative treatment for AR-positive CRPC. And the underlying mechanism for the antineoplastic activity of WNY0824 was also investigated detailedly.
BRD4 regulates AR-mediated transcriptional activity through direct interaction with AR in the nucleus and their recruitment to AR-regulated genes (27). We showed that WNY0824 abrogated enhancer assembly in response to DHT stimulation by displacing both BRD4 and AR from the chromatin, and ultimately silenced AR-regulated genes through inhibiting BRD4. In addition, PLK1 has also been shown to regulate expression levels of AR but the effect of BRD4 is promiscuous (20,27,35). In this study, we observed that JQ1 did not affect AR expression, which was coincident with recent study in CRPC (27), but BI-2536 led to a significant reduction of AR mRNA and AR protein. Thus, we inferred that WNY0824 suppressed AR level through its inhibition of PLK1 but not of BRD4, which might be propitious to the downregulation of the AR pathway.

The ERG gene and the related ETS family member ETV1 is a well-known transcription factor contributing to CRPC progression by augmenting metastasis or resistance to hormone therapy and is currently not tractable (12,37). BRD4 can directly interact with ERG and regulate ERG-mediated transcriptional activity in CRPC cells, which can be partially abrogated by BET inhibitors (27,47). These function of BRD4 in prostate cancer cells may partly explain the reduced expression of ETS-regulated gene CRISP3 in AR-positive CRPC cells after treatment with WNY0824.

MYC is critical to the development of CRPC. Its transcription can be directly regulated by BRD4 which enriched in its distal enhancer (23), and indirectly regulated by BRD4 via affecting the expression of AR-regulated genes ERG and ETV1 which also occupies the distal enhancer of MYC (27). In addition, the stability of MYC was also enhanced by PLK1 via its downregulation of Fbw7-mediated degradation (17). In our studies, MYC was substantial decreased in AR-positive CRPC cells after WNY0824 exposure, accompanied by downregulation of AR as well as upregulation of Fbw7. These observations demonstrated that the transcription of MYC and the stability of its translational product were both impaired by WNY0824. Altogether, the downregulation of MYC should be a comprehensive result of dual inhibition of PLK1 and BRD4.

Both PLK1 and BRD4 are intricately involved in mitosis. We outlined that rather than blocking cells at the G0 phase via inhibition of BRD4, WNY0824 affected cell cycle progression mainly through PLK1 inhibition. PLK1 is an important cell cycle kinase that regulates entry into mitosis, bipolar spindle formation, and the completion of cytokinesis (16). WNY0824 induced a strong and persistent G2/M phase cell-cycle arrest in AR-positive CRPC cells. Furthermore, after treatment with WNY0824, cells exhibited abnormalities in bipolar spindle formation, resulting in the irregular arranging of chromosomes, which led to “mitotic abnormality” and cell death.

Compared with AR-positive cells, AR-negative DU145 and PC3 cell lines exhibited moderate growth inhibition in response to WNY0824. Both AR-positive cells and AR-negative cells displayed comparable mitosis arrest after treatment with WNY0824, which could not explain their different sensitivity to WNY0824. MYC is also important for the growth of AR-negative PC3 and DU145 cells, but BET is not required for transcription of MYC (39). As a result, BRD4 inhibitor failed to
suppress MYC in these CRPC cells. We observed that WNY0824 only enhanced Fbw7-mediated degradation of MYC via PLK1 inhibition but had no effect on MYC transcription and expression in PC3 and DU145 cell lines, leading to negligible downregulation of MYC level, which might explain the insensitivity of DU145 and PC3 to WNY0824.

WNY0824 displayed a moderate antitumor activity in 22RV1 xenograft models at the dose of 60 mg/kg (p.o.). Coincidentally, mechanism of action (MOA) studies found that the effects of WNY0824 on cell cycle related proteins, AR-BRD4-regulated genes, and apoptosis markers were not as remarkable as that in in vitro studies, which might due to the low drug exposure of cancer cells. Further studies with respect to its formulation need to be conduct to improve the oral bioavailability of WNY0824. And further medicinal chemistry studies are also in progress to improve the potency of this chemotype against both PLK1 and BRD4.

In summary, the present data support a new therapeutics strategy for AR-positive CRPC whereby disruption of the AR-transcriptional program, suppression of the ETS pathway and MYC as well as induction of mitotic abnormality via a dual PLK1/BET inhibitor like WNY0824, which also potentially provide a more comprehensive approach to target multiple resistance states. In addition, our results also provide a promising chemotype for the development of new dual PLK1/BET inhibitors as anticancer drugs.

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5. Katsogiannou M, Ziouziou H, Karaki S, Andrieu C, Henry de Villeneuve M,


Figure Legends

**Fig. 1 WNY0824 is a potent dual BET and PLK1 inhibitor.** (A) Chemical structure of WNY0824. (B) Potency of WNY0824 against BET proteins activity. (C) Potency of WNY0824 against PLK1 kinase activity. (D) Heat map showing inhibitory rate against activities of 418 kinds of kinases. The diamond in the black frame represents PLK1. (E) (F) Cellular thermal shift assay of PLK1 (E) and BRD4 (F) in cells with or without WNY0824 incubation. The image (left panel) and quantification (right panel) of the band intensities in immunoblotting. Graphic data were run in triplicate and shown as the mean ± SD.

**Fig. 2 WNY0824 suppresses proliferation of prostate cancer cells and induced apoptosis.** (A) Western blots show expression of PLK1, BET proteins and AR in five prostate cell lines. (B) Prostate cancer cell lines were treated with increasing dose of WNY0824 for 4 days. The cell viability indexes were the percentage of cells contrast to DMSO-treated group. Graphic data were run in triplicate and shown as the mean ± SD. (C) Half-maximum inhibitory concentration (IC₅₀) for WNY0824 in prostate cancer cell lines is shown. Graphic data were run in triplicate and shown as the mean ± SD. (D) LNCaP, VCaP, and 22RV1 cell lines were exposed to graded concentrations of WNY0824 for 96 h, and the live cells were counted every 24h and plotted. Day 0 of drug treatment was set at 1. Graphic data were run in triplicate and shown as the mean ± SD. (E) LNCaP, VCaP, and 22RV1 cell lines were exposed to WNY0824 for 48 h and were detected by FCM after Annexin V/PI staining. Quantification was shown in the right panel. Graphic data were run in triplicate and shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test. (F) Immunoblotting analyses of apoptosis related protein levels, including Mcl-1, Bel-2, cleaved PARP, and cleaved caspase-3 in LNCaP, VCaP, and 22RV1 cells after treated with WNY0824 for 48 h.

**Fig. 3 WNY0824 disrupts AR-transcriptional program.** (A) qRT-PCR analysis of AR in LNCaP, 22RV1, and VCaP cells treated with JQ1, BI-2536, and varying concentrations of WNY0824 for 24 h. Graphic data were run in triplicate and shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test. (B) Western blot analysis of AR levels in VCaP and 22RV1 cells treated with JQ1, BI-2536, and graded concentrations of WNY0824 for 48 h. (C) WNY0824 inhibits AR (left panel) and BRD4 (right panel) occupancy at the PSA and TMPRSS2 enhancer. Graphic data were run in triplicate and shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test. (D) Reduction of PSA transcripts by WNY0824 in LNCaP, 22RV1, and VCaP cells treated for 24 hours. Graphic data were run in triplicate and shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test. (E) Western blots analysis of PSA levels in LNCaP and VCaP cells treated with WNY0824 for 48 h. (F) qRT-PCR analysis of AR-regulated genes in LNCaP, 22RV1, and VCaP cells treated with varying concentrations of WNY0824 for 24 h. Graphic data were run in triplicate and
shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test.

Fig. 4 WNY0824 suppresses ETS pathway and decreases MYC level. (A) (B) qRT-PCR analysis of CRISP3 (A) and MYC (B) in LNCaP, 22RV1, and VCaP cells treated with JQ1, BI-2536, and varying concentrations of WNY0824 for 24 h. Graphic data were run in triplicate and shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test. (C) Western blots analysis of Fbw7 and MYC protein levels in LNCaP, 22RV1, and VCaP cells treated with JQ1, BI-2536, and WNY0824 for 48 h.

Fig. 5 Influence of WNY0824 on cell cycle. (A) Cell cycle analysis of LNCaP, 22RV1, and VCaP cells after treated with JQ1, BI-2536, and WNY0824 for 24 h. Date represents three independent experiments. (B) Immunoblotting analyses of key mitotic related proteins in LNCaP, 22RV1, and VCaP cells after treated with JQ1, BI-2536 and, WNY0824 for 24 h. (C) 22RV1 and VCaP cells were synchronized at the G1/S boundary by double thymidine arrest and released in DMSO or WNY0824 (9 μM) for 14 h, the spindles were determined by immunostaining with α-tubulin (green), and DNA was marked by DAPI (blue).

Fig. 6 In-vivo efficacy of WNY0824 in 22RV1 xenograft models. (A) (B) (C)NOD-SCID mice bearing 22RV1 (n = 5) were orally treated with vehicle control, enzalutamide, or WNY0824 once every day for 18 days. Mean tumor volumes ± SEM (A), weight of tumors from sacrificed mice (Horizontal lines represent the means ± SEM) (B), and mean mouse weight ± SEM (C) are shown, *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test. (D) qRT-PCR analysis of PSA, MYC, TMPRSS2, and BMPR1B in tumor tissues from groups treated with vehicle control and WNY0824 (60 mg/kg). Graphic data were run in triplicate and shown as the mean ± SD. (E) The tumor tissues from groups treated with vehicle control and WNY0824 (60 mg/kg) were immunoblotted with antibodies to detect CDK1, cyclin B1, MYC, cleaved PARP, and GAPDH. Graphic data were shown as the mean ± SD. (F) Tumor tissues from 22RV1 xenograft treated with vehicle control and WNY0824 (60 mg/kg) for 18 days were immunohistochemically analyzed with anti-cleaved caspase-3 and anti-Ki-67 antibodies (n = 5). Representative images are shown. Graphic data were shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 by two-tailed Student's t-test.
Figure 1

A

B

C

D

E

F

The Inhibition Radio of WNY0824 at 1μM

The % inhibition is dependent on the log concentration of WNY0824.

- **BRD2**
- **BRD4**
- **BRD3**
- **BRDT**

- IC_{50} = 402.5 nM
- IC_{50} = 150.7 nM
- IC_{50} = 109.3 nM
- IC_{50} = 311.9 nM

The % inhibition is dependent on the log concentration of WNY0824.

- **PLK1**

- IC_{50} = 22.3 nM

The % inhibition is dependent on the log concentration of WNY0824.

- **DMSO**
- **WNY0824 10μM**
- **WNY0824 50μM**

The % inhibition is dependent on the log concentration of WNY0824.

- **DMSO**
- **WNY0824 10μM**
- **WNY0824 50μM**
Figure 2

A

[Immunoblots of various prostate cancer cell lines showing expression levels of PLK1, BRD4, BRD2, BRD3, AR, and GAPDH]

B

[Graph showing cell viability (% of DMSO) against concentration of WNY0824 (µM) for LnCap, 22RV1, VCaP, DU145, and PC-3 cells]

C

[Graph showing IC₅₀ values for WNY0824 against LnCap, 22RV1, VCaP, DU145, and PC-3 cells]

D

[Graphs showing cell proliferation (Fold Change) over days for LnCap, VCaP, and 22RV1 cells treated with DMSO, WNY0824 (0.1 µM), WNY0824 (1 µM), and WNY0824 (10 µM)]

E

[Flow cytometry analysis showing Annexin V and PI staining for LnCap, VCaP, and 22RV1 cells treated with WNY0824 at concentrations of 0, 0.3, 1, 3, and 9 µM]

F

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Figure 4

A  CRISP3 mRNA

B  MYC mRNA

C  LNCaP

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Fbw7  MYC  GAPDH

22RV1

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Fbw7  MYC  GAPDH

VCaP

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Fbw7  MYC  GAPDH
Figure 5

A

LNCaP

S

G2/M

G0/G1

Percent of total cells [%]

22RV1

S

G2/M

G0/G1

Percent of total cells [%]

VCaP

S

G2/M

G0/G1

Percent of total cells [%]

B

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B

22RV1

VCaP

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Figure 6

A

Tumour Volume (mm³)

Days of Drug Treatment

B

Tumour Weight (g)

Days of Drug Treatment

C

Mouse Weight (g)

Days of Drug Treatment

D

Percentage expression

Vehicle

WNY0824 (60 mg/kg)

E

Vehicle

WNY0824 (60 mg/kg)

CDK1
cyclin B1
MYC
cleaved PARP
GAPDH

Fold Change to Vehicle

Vehicle

WNY0824 (60 mg/kg)

F

Vehicle

WNY0824 60mg/kg

cleaved caspase3

Ki-67

Percentage expression

Vehicle

WNY0824 (60mg/kg)
Molecular Cancer Therapeutics

Novel dual BET and PLK1 inhibitor WNY0824 exerts potent anti-tumor effects in CRPC by inhibiting transcription factor function and inducing mitotic abnormality

Ying Xu, Qianqian Wang, Kunjie Xiao, et al.

Mol Cancer Ther Published OnlineFirst March 27, 2020.

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