Inhibition of the erythropoietin-producing receptor EPHB4 antagonizes androgen receptor overexpression and reduces enzalutamide resistance

Prostate cancer (PCa) cells heavily rely on an active androgen receptor (AR) pathway for their survival. Enzalutamide (MDV3100) is a second-generation antiandrogenic drug that was approved by the Food and Drug Administration in 2012 to treat patients with castration-resistant prostate cancer (CRPC). However, emergence of resistance against this drug is inevitable, and it has been a major challenge to develop interventions that help manage enzalutamide-resistant CRPC. Erythropoietin-producing human hematocellular (Eph) receptors are targeted by ephrin protein ligands and have a broad range of functions. Increasing evidence indicates that this signaling pathway plays an important role in tumorigenesis. Overexpression of EPH receptor B4 (EPHB4) has been observed in multiple types of cancer, being closely associated with proliferation, invasion, and metastasis of tumors. Here, using RNA-Seq analyses of clinical and preclinical samples, along with several biochemical and molecular methods, we report that enzalutamide-resistant PCa requires an active EPHB4 pathway that supports drug resistance of this tumor type. Using a small kinase inhibitor and RNAi-based gene silencing to disrupt EPHB4 activity, we found that these disruptions re-sensitize enzalutamide-resistant PCa to the drug both in vitro and in vivo. Mechanistically, we found that EPHB4 stimulates the AR by inducing proto-oncogene c-Myc expression. Taken together, these results provide critical insight into the mechanism of enzalutamide resistance in PCa, potentially offering a therapeutic avenue for enhancing the efficacy of enzalutamide to better manage this common malignancy.

According to the latest cancer statistics, prostate cancer (PCa) continues to be one of the biggest threat to human health, ranking as the third leading diagnosed type of cancer and the sixth leading cause of death (1). It has been well-established that androgen receptor (AR) signaling plays a major role in maintaining PCa’s survival and proliferation (2). Thus, the AR pathway serves as the “keystone” of disease progression. As a result, the androgen deprivation therapy (ADT) remains to be the major treatment against PCa (3). However, nearly all patients eventually progress to a stage called castration-resistant prostate cancer (CRPC) due to the generation of resistance to androgen depletion (4). Enzalutamide (MDV3100) is a second-generation antiandrogen drug. Due to its much higher potency to block AR signaling and better therapeutic effect (5), enzalutamide was approved by FDA in 2012 to treat CRPC. Unfortunately, resistance still arises after several months’ medication (6). Thus, how to overcome the enzalutamide-resistant CRPC is among the most urgent issues in clinic. Erythropoietin-producing human hematocellular (Eph) receptors have a broad range of functions upon binding to ephrins, which are the ligands of Eph receptors (7). Both Eph receptors and ephrins are transmembrane proteins that usually exert their effects by direct cell-to-cell contact. All Eph receptors contain an N-terminal ligand-binding domain, a juxtamembrane region, and a C-terminal intracellular kinase domain (8). Binding of Eph receptors to ephrins triggers the downstream responses that are involved in various biological processes such as cell movement and angiogenesis (7–9). There is increasing evidence to show that Eph–ephrin signaling is closely related to tumorigenesis, among of which EphB4-ephrinB2 is one of the most widely studied pairs (8–10). Overexpression of EphB4 has been implicated in multiple types of cancers, such as breast cancer (11, 12), ovarian cancer (13), colon cancer (14, 15), PCa (16, 17), and melanoma (18, 19). Such dysregulation is often associated with poor prognosis and malignant change in terms of proliferation, invasion, and metastasis. Although EphB4 is well-documented in PCa, whether the signaling is involved in enzalutamide resistance is unknown. Herein, we show that...
enzalutamide-resistant PCa entails activation of the EphB4 pathway to ensure their drug-resistant property. Application of small kinase inhibitor and RNAi to disrupt the EphB4 activity re-sensitizes enzalutamide-resistant PCa to the treatment both in vitro and in vivo, which can be attributed to the suppressive effect on AR. In short, the data we present offer a new approach to enhance the efficacy of enzalutamide.

Results

Up-regulation of AR correlates with an active state of EphB4 in enzalutamide-resistant CRPC

To dissect the underlying mechanisms that are responsible for the acquisition of enzalutamide resistance, we performed a standard RNA-seq analysis using four cell lines in-house: LNCaP, MR49F, C4-2, and C4-2R. Whereas LNCaP and C4-2 are enzalutamide-sensitive PCa cells, MR49F and C4-2R cells are enzalutamide resistant but are derived from LNCaP and C4-2, respectively. Upon comparing the RNA profiles of drug-resistant cell lines to their drug-sensitive counterparts, 1268 genes were identified as differentially expressed genes in C4-2R and 903 genes were found as differentially expressed genes in MR49F (Tables S1A and S1B), which is in agreement with our published results in C4-2R (20). Using KEGG predefined pathway analysis, pathways related to PCa progression were significantly enriched in C4-2R and MR49F (Fig. 1A), indicating that enzalutamide-resistant CRPC becomes more malignant and...
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mimics the very late stage of PCa. Consistent with the published results, AR was still active in MR49F, as revealed by the gene set enrichment analysis (GSEA) and heat map of AR target genes (Fig. 1, B and D, and Table S2A). The active state of AR was further validated by monitoring the mRNA and protein levels of AR (Fig. 1, F and G), implying that AR reactivation is essential to confer resistant property on cells. To investigate the “accomplice” behind AR reactivation, we searched our results of ingenuity pathway analysis and identified multiple signaling networks that were highly active in enzalutamide-resistant cell lines, among which the axon guidance pathway ranked on the top both in C4-2R (Fig. 1, C and E, and Tables S2B and S3A) and MR49F (Table S3B). Axon guidance is a complex process that directs the growth of neurons and helps axons to locate the right position with the aid of many signal molecules, but recent studies have revealed its novel role in cancer development (21, 22). We performed a series of tests and found that the phosphorylation level of EphB4, an important signal molecule in axon guidance pathway, was much higher in C4-2R and MR49F compared with LNCaP and C4-2 (Fig. 1H). This indicates the activation of EphB4 in enzalutamide-resistant CRPC. Taken together, these results suggest that AR continues to be functional in enzalutamide-resistant CRPC, which couples with the abundant phosphorylation of EphB4. Thus, activation of the EphB4 pathway might be associated with the acquisition of drug resistance.

Blockade of EphB4 by kinase inhibitor re-sensitizes enzalutamide-resistant CRPC to treatment

Next, we then tested their sensitivity to NVP-BHG712, a specific kinase inhibitor of EphB4 that had been described elsewhere (23). Not surprisingly, MR49F and C4-2R were much more vulnerable to inhibitor treatment, as denoted by lower IC_{50} compared with LNCaP and C4-2 (Fig. 2, A and B). The sensitivity to EphB4 inhibitor was further validated by cell growth assay (Fig. 2, C and D). Although application of the EphB4 inhibitor could slightly suppress cell proliferation of LNCaP, that effect was not as strong as enzalutamide. However, the effects of these inhibitors on MR49F were dramatically different, as enzalutamide hardly inhibited cell proliferation, whereas EphB4 inhibitor almost completely impeded the increase of cell number. Interestingly, the inhibitory effect on MR49F could be augmented by co-treatment with enzalutamide. However, that was not delineated in LNCaP, as the combination treatment was not better than single treatment with enzalutamide. Furthermore, a combination treatment could dramatically trigger apoptosis much stronger than any of the single therapies in all four cell lines as indicated by cleaved-PARP, a marker of apoptosis (Fig. 2F). However, this provides a more promising expectation toward bypassing the enzalutamide resistance, as MR49F and C4-2R cells are not responsive to enzalutamide treatment but will be subject to cell death when the EphB4 inhibitor is applied. In addition, to achieve a similar level of cell death, we need to use much higher concentrations of EphB4 inhibitor in LNCaP and C4-2. Finally, co-targeting AR and EphB4 using these two drugs sufficiently antagonized the clonogenic capability than any of the single treatments in MR49F and C4-2R (Fig. 2, F–H). To make sure enzalutamide and the EphB4 inhibitor displayed a synergistic effect, we calculated the 50% combination index (CI_{50%}). 50% means that applies half of the IC_{50} of one drug as the background, then calculate the IC_{50} of another drug under this condition: CI = A%/A + *B/B. Although A and B are the original IC_{50} of two drugs, *B is the conditioned IC_{50} when a certain percentage of the IC_{50} of A drug serves as the background. For CI_{50%}, A’ = 0.5A, thus CI_{50%} = 0.5 + *B/B. If CI_{50%} > 1, it indicates two drugs are antagonistic; if CI_{50%} = 1, it indicates two drugs are additive; if CI_{50%} < 1, it indicates two drugs are synergistic. According to our results, two drugs showed synergistic effects in MR49F and C4-2R, as implied by the calculated CI_{50%} (Fig. 2, I and J) and the cell viability curves (Fig. S1, A–D). In summary, these data provide strong evidence that inhibition of EphB4 has a synergistic effect with enzalutamide, which promotes the notion of combination treatment for enzalutamide-resistant CRPC.

Up-regulation of AR by EphB4 contributes to enzalutamide resistance

Next, we asked how the activation of EphB4 was linked to drug resistance. Previously it had been reported that up-regulation of AR was sufficient to endow LNCaP with an androgen-resistant trait (24). Our data suggest that in enzalutamide-resistant CRPC, AR acquires a further increase together with an active EphB4 signaling, which made us consider that EphB4 may serve as a positive regulator of AR. To test our hypothesis, we treated enzalutamide-resistant CRPC with EphB4 inhibitor and found that ablation of EphB4 signaling indeed was sharply down-regulated in AR (Fig. 3, A and B). That effect was due to the reduction of mRNA (Fig. 3A) but not enhanced protein degradation (data not shown). To better reflect the activity of AR, we tested the mRNA and protein levels of several canonical AR target genes (PSA, FKBP5, and NKX3.1). Consistent with our expectation, a single treatment was not sufficient to block the expression of these genes, but a combination treatment tremendously reduced AR target genes in both C4-2R and MR49F (Fig. 3, C–H). Although enzalutamide or EphB4 inhibitor per se was unable to completely interfere with the transcriptional ability of AR, a combination treatment displayed a synergistic effect, as the protein levels of PSA in C4-2R and MR49F largely disappeared (Fig. 3, C, D, F, and G). To further validate our hypothesis, we treated LNCaP and C4-2 with recombinant human ephrinB2-Fc. As indicated by the phosphorylation of receptor, ephrinB2-Fc was effective in switching EphB4 to an active state, and this effect was disrupted by the EphB4 inhibitor (Fig. 3I). More importantly, the active EphB4 led to the elevation of AR and improved cell survival under enzalutamide treatment (Fig. 3, J and K). Based on these results, we conclude that EphB4 positively regulates AR, which is responsible for the acquisition of enzalutamide resistance.

Depletion of EphB4 mimics the effect of EphB4 inhibitor and offsets enzalutamide resistance

To rule out the off-target effect of the EphB4 inhibitor, we performed RNAi experiments in C4-2R. Upon knockdown of EphB4, both AR and PSA levels were attenuated (Fig. 4A). Of note, an additional treatment of enzalutamide led to a further
Figure 2. Inhibition of EphB4 overcomes enzalutamide resistance. A and B, three-day cell viability assays to compare the IC50 of the EphB4 inhibitor between MR49F and LNCaP, C4-2R, and C4-2. Data were normalized to groups without any treatment and shown as mean ± S.D. (n = 3). The plot is from one representative experiment of three repeats. C and D, six-day cell growth assays of LNCaP and MR49F upon addition of 10 μM enzalutamide (Enz), NVP-BHG712 (NVP), or both drugs (E + N). The concentration of NVP is 2.5 μM for LNCaP and 0.25 μM for MR49F. Culture medium with new drugs was refreshed at day 3. Data were normalized to day 0 and denoted as mean ± S.D. (n = 3). The experiment was repeated twice and one representative was shown. E, apoptosis assay of four cell lines. Upon 48 h administration of the indicated drugs, cells were subjected to IB against cleaved PARP. Results are representatives of three replicates. F and G, clonogenic assay of C4-2R and MR49F with the indicated drug concentrations for up to 14 days. Medium with new drugs was replaced at day 7. H, quantification of F and G. Data are mean ± S.D. of three replicates. I and J, calculation of CI50%, cells were cultured either with or without half IC50 of another drug for 3 days, then calculate its own IC50 or *IC50. The statistical method used is two-tailed t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 3. EphB4 contributes to enzalutamide resistance by up-regulating AR. A and B, qPCR and IB to measure the mRNA and protein expression of AR in C4-2R and MR49F treated with the indicated drugs for 24 h. The concentration of NVP used in qPCR is 1.5 and 0.25 μM for C4-2R and MR49F, respectively. Data of qPCR were normalized to control groups and shown as mean ± S.D. (n = 3). Results of A and B are representative of three independent experiments. C and F, IB to detect the protein expression of AR and PSA in C4-2R and MR49F treated with the indicated drugs for 48 h. Images of IB are representative of three experiments. D and G, quantification of three independent PSA in C and F. E and H, qPCR to detect the mRNA level of AR and its target genes. Cells were treated with the indicated drugs for 24 h. The concentrations of enzalutamide and NVP are the same as in C and F. Data of qPCR were normalized to control groups. The graphs of qPCR are one experiment of three repeats and shown as mean ± S.D. (n = 3). I, IP (anti-EphB4) and IB (anti-phosphotyrosine) to test the active state of EphB4 after LNCaP and C4-2 were treated with 1 μg/ml of ephrinB2-Fc (B2) for 15 min, with or without preincubation with NVP for 30 min. J, IB against AR in LNCaP and C4-2 after 24 h application of 1 μg/ml of B2. Results are from one of three duplicates. K, six-day cell growth assay of LNCaP upon treatment of 10 μM Enz, 1 μg/ml of B2 or both. Drugs were refreshed at day 3. Results were normalized to day 0 and exhibited as mean ± S.D. (n = 3). One of triplicates was rendered. The statistical method used is two-tailed t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
reduction of PSA (Fig. 4). In agreement with results obtained with the EphB4 inhibitor, cells with depletion of EphB4 became more sensitive to enzalutamide-induced apoptosis and growth inhibition (Fig. 4, C and D), although the effect on apoptosis was not that ideal. Ablation of EphB4 also enhanced the suppressive effect of enzalutamide on colony formation (Fig. 4, E and F). Furthermore, some results were further justified in MR49F (Fig. 4, G and H). In summary, these data provide another set of evidence that EphB4 is a *bona fide* regulator of AR whose inhibition re-sensitizes cells to enzalutamide.

**Inhibition of EphB4 quenches enzalutamide resistance via down-regulating AR-V7**

There are sufficient evidence to link the AR splice variants with enzalutamide resistance (25, 26), of which the most important one is AR-V7. To test whether EphB4 regulates AR-V7 as...
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Well, we switched to the 22Rv1 cell line, which has a high expression level of AR-V7 and is resistant to enzalutamide. As indicated, inhibition of EphB4 reduced expression levels of both full-length AR (AR-FL) and AR-V7 (Fig. 5, B and E). Although we did not observe a decrease of NkX3.1, the decline of PSA and FKBP5 was still recapitulated. Combination treatments also greatly suppressed their expression and showed a synergistic effect to inhibit AR-FL (Fig. 5, A–C). To better reflect the activity of AR-V7, we tested the expression levels of PLK1, UBE2C, and CDC25C, which are known as AR-V7-regulated genes (27). As expected, enzalutamide did not perturb the expression of these genes, but application of the EphB4 inhibitor clearly reduced their expression (Fig. 5, D and E). Furthermore, disruption of EphB4 signaling was able to induce apoptosis in 22Rv1 (Fig. 5F). Although apoptosis was not multiplied by additional treatments of enzalutamide, co-treatment with two drugs dramatically suppressed cell proliferation (Fig. 5G). Finally, depletion of EphB4 faithfully recapitulated these results as designated by the reduction of AR and their target genes (Fig. 5, H and J), as well as impaired cell growth once enzalutamide was added (Fig. 5F). Collectively, these results support the conclusion that inhibition of EphB4 also overcomes enzalutamide resistance by counteracting the function of AR-V7.

c-Myc is responsible for the induction of AR by EphB4

To explore how EphB4 controls the expression of AR, we scanned many possible transcription factors that might regulate AR (28), and found that c-Myc and its related pathway were highly up-regulated in resistant cells (Fig. S2A and Table S2C). Indeed, resistant cells displayed higher c-Myc activity as verified by a c-Myc reporter assay and qPCR testing of c-Myc target genes (Fig. 6, B–D). Inhibition of EphB4 reduced both expression of AR and c-Myc in all three resistant cell lines (Fig. 6E, and Fig. S2, B and C). To directly depict the effect of c-Myc, we treated cells with a c-Myc-specific inhibitor and observed the reduction of AR (Fig. 6F, and Fig. S2, D and E), indicating that EphB4 might regulate AR via c-Myc. To further affirm our hypothesis, we treated LNCaP and C4-2 with ephrinB2-Fc. As shown here, activation of EphB4 increased both AR and c-Myc (Fig. 6G), and this phenotype was reversed by an additional treatment of c-Myc or EphB4 inhibitor (Fig. 6H). In addition, overexpression of c-Myc elevated AR expression, which was neutralized by c-Myc inhibitor (Fig. 6I). Moreover, depletion of EphB4 ablated expression of AR and c-Myc (Fig. 6J, and Fig. S2, F and G) was rescued by add-back of EphB4 (Fig. 6K, and Fig. S2, H and J). Besides, after re-expression of EphB4, the sensitivity to enzalutamide was also restored (Fig. 6L, and Fig. S2, J and K). To confirm that our findings were consistent with the clinical situations, we obtained four available datasets of patients’ samples from the cBioportal database and carried out correlation analysis. The results showed that there was a strong correlation between EphB4 and c-Myc, c-Myc and AR, as well as EphB4 and AR, especially in metastatic CRPC samples (Fig. 6, M–O, and Fig. S3, A–C, G–J, and M–O). In comparison, this relationship was not significant in normal tissues (Fig. S3, D–F and J–L), which suggests that the EphB4/c-Myc axis may be responsible for the induction of AR. In sum, these data support the conclusion that the regulation of AR by EphB4 is through the modulation of c-Myc.

Co-targeting AR and EphB4 overcomes enzalutamide resistance in 22Rv1 xenograft model

To further explore the translational merit of our finding, we carried out an in vivo 22Rv1 xenograft experiment. As expected, monotherapy with enzalutamide or EphB4 inhibitor was insufficient to delay the tumor growth, whereas a combination treatment effectively decelerated this process (Fig. 7A). After reaching the end point, both the volume and weight were smallest in the combination group (Fig. 7, B and C, and Fig. S4A). This is unlikely due to the toxic effect, as the body weight of mice displayed no dramatic difference at the end of medication (Fig. S4B). To view the histological change of cells, we performed H&E staining and found that in the vehicle or mono-treatment group, there were more cells in mitosis. In contrast, large quantities of apoptotic cells were easily identified in the combination treatment group as shown by the condensed nuclei (Fig. S4C). This was in accordance with the immunofluorescence staining of Ki-67 and cleaved caspase 3 (Fig. 7, D–G), which is the marker of proliferating or apoptotic cells, respectively. In agreement with the in vitro results, staining of AR showed a weaker signal in groups treated with EphB4 inhibitor (Fig. 7, F and G), which was substantiated by qPCR and immunoblot tests of AR-FL and AR-V7 (Fig. 7, H–J). Not surprisingly, c-Myc was also decreased in groups treated with the EphB4 inhibitor (Fig. 7I), which explains the reduction of AR. In addition, the activities of both AR-FL and AR-V7 were largely discontinued in these groups as shown by the down-regulation of their target genes (Fig. 7, H–J). Although the variation among mice was much higher than that in vitro, quantification of PSA from tumors and mice serum still suggests the synergistic effect of these two drugs to inhibit the activity of AR (Fig. 7, K and L). Therefore, these data provide additional evidence that inhibition of EphB4 is sufficient to overcome enzalutamide resistance by down-regulating the expression of AR in vivo, which may point out a new way to treat clinical patients with enzalutamide-resistant CRPC.

Discussion

The generation of drug resistance restricts the long-term usage of enzalutamide to treat CRPC patients (6). Therefore, it is urgent to find the mechanism behind the spontaneous acquisition of enzalutamide resistance. Herein, we demonstrate that activation of EphB4 is one of the major reasons to induce enzalutamide resistance. As a receptor tyrosine kinase, overexpression of EphB4 has been reported in various types of cancers and usually predicts a worse outcome (11–19), but the exact function pattern of EphB4 depends on different cellular contexts. For example, in melanoma and ovarian cancers, active EphB4 promotes tumor progression and thus is defined as a survival factor (13, 19). However, this is not the case in breast cancer, as the activation of EphB4 by its ligand actually suppresses tumor growth by triggering the Abl-Crk pathway (12). Interestingly, these receptors are poorly phosphorylated despite the high expression in advanced breast cancer, and deple-
Figure 5. EphB4 positively regulates AR-V7 in 22Rv1. A and B, qPCR and IB were used to examine AR-FL and its downstream genes. 22Rv1 were treated with 20 μM enzalutamide, 3 μM NVP or both drugs for 24 h in A and 48 h in B. Results of qPCR were normalized to control and exhibited as mean ± S.D. (n = 3) from one of three experiments. IB bands are representative of three experiments. C, quantification of three independent PSA in B. D and E, qPCR and IB to probe AR-V7 and its downstream genes. Cells were treated with the same concentrations of drugs as in A and B for 24 or 48 h. Data of qPCR were expressed as mean ± S.D. (n = 3) from one of three experiments. Western results are examples of three replicates. F and G, a 48-h apoptosis assay and 6-day cell growth assay of 22Rv1 under different treatments. 10 μM enzalutamide and 3 μM NVP were used in G. Data of cell growth were normalized to day 0 and shown as mean ± S.D. (n = 4). All experiments were repeated three times and one of each was provided. H and I, 22Rv1 with stable depletion of EphB4 was subjected to IB against AR-FL, AR-V7, and their target genes. One of three Western blots was shown. J, cell proliferation assay of 22Rv1 with stable EphB4 knockdown. 20 μM enzalutamide was added. Data were normalized to day 0 and expressed as mean ± S.D. (n = 4). Plot was generated from one of three experiments. The statistical method used is two-tailed t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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A. Enrichment plot: c-Myc Pathway

B. Relative c-Myc Activity

C. Relative c-Myc Activity

D. qPCR

E. Enzyme (20μM) vs NVP (1.5 μM) in C4.2R

F. 10074-G5 (1 μM) in C4.2R

G. 10074-G5 (1 μM) in C4.2R

H. B2 (1 μg/ml) vs NVP (1 μM) in C4.2R

I. c-Myc plasmid vs 10074-G5 (1 μM) in C4.2R

J. EphB4 vs C4.2R

K. EphB4 vs C4.2R

L. Cell Growth (C4.2R)

M. mCRPC (SU2C-PCF Dream Team, Cell 2015)

N. mCRPC (SU2C-PCF Dream Team, Cell 2015)

O. mCRPC (SU2C-PCF Dream Team, Cell 2015)
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The function of EphB4 in breast cancer is dichotomous, with the tumor-suppressive effect relying on its regular phosphorylation and the tumor-promoting effect independent of its kinase activity. Therefore, the function of receptor tyrosine kinases may vary based on whether they possess kinase activity or not, providing caution and inspiration to researchers about individual research methods.

Although there are several reports concerning EphB4 in PCA, whether the function depends on its autophosphorylation is not clear. More importantly, the role of EphB4 in acquisition of enzalutamide resistant is completely unexplored. To our knowledge, we are the first to link the function of EphB4 with its kinase activity in PCA. Surprisingly, this phenomenon could not be explained by the amount difference of its ligand or the receptor per se, as the immunoblot actually shows almost the same levels. This implies there are other reasons that contribute to the activation of EphB4, possibly because of different expressions of phosphatase or cross-talk with other pathways as inspired in the case of EphA2 (29, 30). Considering that the relationship of AR to ADT resistance has already been well-established (24), the modulation of AR by EphB4 could serve as the reason to explain the synergistic effect of enzalutamide and EphB4 inhibitor.

In real clinical scenarios, the effect of enzalutamide is often minimized by the existence of AR variants (25, 26), of which the most important one is AR-V7. This type of variant is thought to derive from alternative splicing of cryptic exons and lacks the C-terminal ligand-binding domain (31). As such, the function of AR-V7 does not require androgen and cannot be restricted by enzalutamide, which targets the C terminus of AR. Herein, we show that inhibition of EphB4 is also able to decrease AR-V7 and its activity in 22Rv1, thus re-sensitizing cells to enzalutamide treatment. Many cell cycle-related genes are regulated by AR-V7. For example, PLK1 is a well-known oncogene whose overexpression is able to transform cells and foster tumor progression (32). Given that PLK1 is a downstream target of AR-V7 and also positively modulates AR signaling (33), it is likely that they regulate each other and form a positive feedback loop to promote cancer development. Accordingly, blocking the EphB4 signaling will be extremely powerful to treat advanced CRPC, as both AR signaling and other tumor promoters such as PLK1 will be repressed, eventually enhancing the efficacy of enzalutamide.

Previously, the connection between EphB4 and c-Myc was reported in colon cancer (15), and positive regulation of AR by c-Myc in LNCaP was already indicated elsewhere (34). Thus, our data in enzalutamide-resistant CRPC were consistent with the published results. Nevertheless, how c-Myc is overexpressed in advanced PCA is not well-studied. Our previous publications showed the contribution of β-catenin and PI3K/AKT/mTOR to ADT resistance (20, 35). Considering that these two pathways are known to elevate c-Myc (36, 37), and EphB4 acts upstream of β-catenin and PI3K/AKT/mTOR (7–9), it is reasonable to extrapolate that EphB4 and c-Myc work as the pivot to control the expression of AR through multiple signaling pathways in enzalutamide-resistant CRPC. Further study will need to focus on enzalutamide-resistant samples to confirm this causal link in clinic.

Until now, there are several EphB4 inhibitors being tested in clinic trials (38). Our data support the notion to utilize the EphB4 inhibitor in cancer treatment, especially in enzalutamide-resistant CRPC. The IC_{50} values of the EphB4 inhibitor for resistant cells are much lower, which makes it less likely to have an off-target effect or induce toxicity. Furthermore, given the fact that AR variants are very common in patients medicated with enzalutamide, drugs that reduce the expression of AR variants will have more advantages over other candidates. In summary, we show that activation of EphB4 is one mechanism to reactivate AR signaling, thus inhibition of EphB4 enhances the efficacy of enzalutamide.

**Experimental procedures**

**Cell culture**

LNCaP, MR49F, C4-2, C4-2R, and 22Rv1 cells were cultured in 100-mm^2 tissue culture dish using RPMI 1640 medium with 10% fetal bovine serum, 5% penicillin-streptomycin under the conditions of 37 °C and 5% CO₂. LNCaP, MR49F, and C4-2R cell lines were purchased from ATCC (Manassas, VA). MR49F is a enzalutamide-resistant derivative of LNCaP obtained from Dr. Amina Zoubeidi (39), whereas C4-2R is derived from C4-2 and kindly provided by Dr. Allen Gao (40). The concentration of enzalutamide for maintenance of MR49F and C4-2R was 10 or 20 μM, respectively. Before any experiment, enzalutamide was removed for 48 h.

**Cell proliferation assay**

3 × 10³ cells were seeded per well onto 96-well-plates overnight with RPMI 1640. On the second (day 0) and third days, fresh new medium either with or without drugs was added to cells. At days 0, 3, and 6, MTS reagent (Biovision, Milpitas, CA, catalog number K300-2500) or AquaBluer (MultiTarget Phar-
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A

Volume (mm^3)

3000

2000

1000

0

0

5

10

15

Days

Vehicle

Enz

NVP

E + N

B

Tumor Weight (g)

3

2

1

0

Vehicle

Enz

NVP

E + N

C

Vehicle

Enz

NVP

E + N

D

Ki-67

Merged

Vehicle

Enz

NVP

E + N

E

Cleaved Caspase 3

Merged

Vehicle

Enz

NVP

E + N

G

IF Quantification

Kl-67

Cleaved Caspase3

AR

Positive Cells (%)

0.00

0.25

0.50

0.75

1.00

Vehicle

Enz

NVP

E + N

F

AR

Merged

Vehicle

Enz

NVP

E + N

H

qPCR (tumor)

Relative Level

2.0

1.5

1.0

0.5

0.0

AR-FL

PSA

FKBP5

NKK3.1

Vehicle

Enz

NVP

E + N

I

qPCR (tumor)

Relative Level

2.0

1.5

1.0

0.5

0.0

AR-V7

PLK1

UBE2C

CDC25C

Vehicle

Enz

NVP

E + N

J

# of tumor

Vehicle

Enz

NVP

E + N

AR-FL

AR-FL/Actin

AR-V7

AR-V7/Actin

c-Myc

Myc/Actin

PSA

PSA/Actin

PLK1

PLK1/Actin

CDC25C

CDC25C/Actin

β-Actin

150kDa

100kDa

100kDa

75kDa

75kDa

50kDa

50kDa

75kDa

75kDa

50kDa

37kDa

25kDa

25kDa

150kDa

100kDa

100kDa

Freeze

100kDa

100kDa

75kDa

75kDa

50kDa

50kDa

37kDa

25kDa

25kDa

50kDa

50kDa

PSA quantification (Tumor)

Relative Level

1.5

1.0

0.5

0.0

Vehicle

Enz

NVP

E + N

Serum PSA

Relative Level

1.5

1.0

0.5

0.0

Vehicle

Enz

NVP

E + N

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maceuticals LLC, Colorado Springs, CO, catalog number 6015) was added to 96-well-plates and incubated for 1–4 h. The OD value at 490 nm (MTS) or excitation 540/emission 590 fluorescence (Aquabluer) was obtained using GloMax Discover plate reader (Promega, Madison, WI).

**Clonogenic assay**

1 × 10³ cells were seeded per well onto 6-well-plates and cultured overnight. The next day, the drug and culture were directly applied for up to 14 days. The drugs were refreshed at day 7. Upon reaching the end point, cells were fixed with 10% formalin for 10 min and washed once with clean PBS. Right after the PBS washing step, cells were stained by 0.5% crystal violet solution (0.5 g of crystal violet, 20 ml of methanol in 100 ml of ddH₂O) for 30 min and washed three times with tap water. The plates were then air-dried and subject to pictures taken by ChemiDoc Imaging System (Bio-Rad).

**qPCR**

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) from tissues or cells. Reverse transcription of total RNA was performed with SuperScript IV First-Strand Synthesis System (Thermo, Waltham, MA, catalog number 18091200). qPCR were carried out using PowerUp SYBR Green Master Mix (Thermo, A25741) and QuantStudio 5 Real-Time PCR System (Thermo).

**RNAi and transfection**

Cells were infected by lentivirus overnight with either the empty vector or shRNAs targeting the indicated genes. After the infection fresh medium with puromycin was added to select the infected cells for subsequent culture and experiments.

pCMV4a-Flag-c-Myc was a gift from Hening Lin ([RRID:Addgene_102625](https://www.addgene.org/addgene/102625)), whereas FUW-ubiquitin-EphB4-SV40-GFP (B4G) was a gift from Eduard Batlle ([RRID:Addgene_65444](https://www.addgene.org/addgene/65444)). Transfection was performed using jetPRIME® Versatile DNA/siRNA transfection reagent (Polyplus-transfection, illkirch, France, catalog number 114-15) according to the manufacturer’s instruction.

**c-Myc reporter assay**

c-Myc firefly luciferase reporter vector (pGL4[luc2P/Myc/Hygro], CS 180201) and control Renilla luciferase vector (pRL-TK, E2241) were purchased from Promega. 3 × 10⁴ cells were seeded onto 24-well-plates and co-transfected with two luciferase vectors in a ratio of 1:50 (Renilla:firefly). After 24 h culture, experiments was performed using a Dual Luciferase® Reporter Assay System (Promega, E1910). The luminescence was viewed by GloMax Discover plate reader.

**Xenograft experiments**

All the animal experiments performed were approved by the University of Kentucky Division of Laboratory Animal Resources. The formulation of drugs are indicated below: enzalutamide was ground to fine particles and dissolved in a solvent mixture containing 1% carboxymethyl cellulose, 0.1% Tween 80, and 5% DMSO; NVP-BHG 712 was dissolved in a solvent mixture containing 10% N-methyl-2-pyrrolidone and 90% PEG 300 (PEG300). 22Rv1 cells (2.5 × 10⁵ cells/mouse) were inoculated into castrated athymic nude mice (Hsd: Athymic Nude-Foxn1nu, Envigo). When the average tumor size reached 100 mm³, animals were randomized into four groups followed with *per os* medication. The group setting is: Group 1 was treated with vehicle (1% carboxymethyl cellulose, 0.1% Tween 80, and 5% DMSO + 10% N-methyl-2-pyrrolidone and 90% PEG300) every day; Group 2 was treated with 25 mg/kg of enzalutamide every day; Group 3 was treated with 25 mg/kg of NVP-BHG 712 every 2 days; and Group 4 was treated with 25 mg/kg of enzalutamide (every day) and 25 mg/kg of NVP-BHG 712 (every 2 days). Measurement was done by caliper every 2 days, and tumor volumes were calculated with the formula: \( V = (L \cdot W \cdot H)/2 \), where \( V \) is volume (mm³), \( L \) is length (mm), \( W \) is width (mm), and \( H \) is height (mm). The experiment was stopped whenever the average tumor volume reached 2000 mm³, and all mice were euthanized immediately followed with blood collection from the heart. Parts of tumors were cut off and stored in liquid nitrogen for protein and RNA extraction, with the remainder fixed with 10% formalin and subject to slides preparation.

**Serum PSA detection**

After collecting blood from euthanized mice, samples were centrifuged at 2000 rpm for 3 min. Supernatants were transferred to clean Eppendorf tubes and stored in –80 °C until used in the experiment. Serum PSA level was quantified with a PSA (Human) ELISA Kit (Abnova, Taiwan, catalog number KA0208) according to the manufacturer’s instruction.

**Statistical analysis**

All Western bands were quantified using Image Lab software (Bio-Rad). The significance of the results was analyzed using either two-tailed unpaired Student’s *t* test or one-way analysis of variance by GraphPad Prism 8 statistical software, depending on different experimental settings. A *p* value less than 0.05 indicates statistical significance. Please see supporting information for complete details.

**Data availability**

All data are contained within the article and supporting material.

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**Figure 7. In vivo 22Rv1 xenograft experiment.** A, 14-day tracking of tumor growth. Measurement was made every 2 days. The tumor size was calculated by \( V = (L \cdot W \cdot H)/2 \). B, final weight of tumors when medication was stopped. C, real picture of tumors when harvesting. D–F, Immunofluorescent staining of Ki-67, cleaved caspase 3, and AR of tumor slices (×10 magnification). Scale bars, 25 μm. G, quantification of D–F. Results are mean ± S.D. from three mice of each group. H–J, qPCR and immunoblot to examine the expression of AR-FL, AR-V7, and their downstream targets. Data of qPCR were normalized to the vehicle group. Results were obtained from three mice of each group. K, quantification of PSA from three mice in J, L, ELISA to test serum PSA. Data were acquired from three mice of each group and normalized to vehicle control. The statistical method used is two-tailed *t* test. *p < 0.05; **, *p < 0.01; ***., *p < 0.001.
Inhibition of EphB4 overcomes enzalutamide resistance

Author contributions—C. L., N. A. L., and D. H. data curation; C. L., N. A. L., and D. H. formal analysis; C. L. validation; C. L. investigation; C. L. visualization; C. L. writing-original draft; C. L., Y. K., F. M., and E. F. project administration; N. A. L., D. H., Y. Z., J. L., C. W., and X. L. methodology; Y. Z., J. L., C. W., and X. L. supervision; C. W., Q. W., and X. L. resources; X. L. conceptualization; X. L. software; X. L. funding acquisition; X. L. writing-review and editing.

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