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Cytotoxic and Apoptotic Agent Encorafenib Controversially Alters Invasive Properties of Castration-Resistant Prostate Cancer Cells with High and Moderate Metastatic Potential

Sitotoksik ve Apoptotik Ajan Encorafenib, Yüksek ve Orta Derecede Metastatik Potansiyele Sahip Kastrasyona Dirençli Prostat Kanseri Hücrelerinin İnvaziv Özelliklerini Tartışmalı Şekilde Değiştirir

[1]Isil Ezgi Eryilmaz, [2]Ceyda Colakoglu Bergel, [2]Bilge Arioz, [1]Unal Egeli

[1]Bursa Uludag University, Faculty of Medicine, Department of Medical Biology, Bursa, Türkiye
[2]Bursa Uludag University, Institute of Health Sciences, Department of Medical Biology, Bursa, Türkiye

ORCID ID of the authors
IEE. 0000-0002-3316-316X
CCB. 0000-0002-7471-5071
BA. 0009-0009-3591-1561
ÜE. 0000-0001-7904-883X

Correspondence / Sorumlu yazar:
Işıl Ezgi ERYILMAZ

Bursa Uludag University, Faculty of Medicine, Department of Medical Biology, Bursa, Türkiye

e-mail: ezgieryilmaz@uludag.edu.tr

Abstract: Metastatic castration-resistant prostate cancer (mCRPC) is a highly aggressive form of prostate cancer (PCa) with limited treatment options and poor prognosis. *BRAF* mutations, although rare, contribute to the progression of PCa by activating the MAPK signaling pathway, which is implicated in cellular proliferation, survival, and metastasis. In this study, we first investigated the potential anticancer and anti-invasive effects of Encorafenib (Enco), a second-generation BRAF inhibitor, in mCRPC cell lines with varying metastatic potentials: moderate metastatic DU145 (*BRAF*-mutated) and high metastatic PC3 (*PTEN*-null). Our results showed that Enco reduced cell viability and induced apoptosis in both cell lines in a concentration- and time-dependent manner, with DU145 cells being more sensitive. While Enco inhibited migration in PC3 cells, it had no significant effect on the migration of DU145 cells. Furthermore, Enco treatment increased the expression of genes related to angiogenesis and invasion (*VEGF-a*, *HIF1-a*, *MMP9*, and *MMP2*) in both cell lines. These findings suggest that while Enco may have potential as a cytotoxic agent for mCRPC, its effects on migration, invasion, and gene expression may vary based on the specific genetic alterations of the cancer cells. This highlights the need for personalized treatment strategies and the potential for adaptive resistance mechanisms. Further studies, particularly combination therapies targeting multiple signaling pathways, are necessary to improve the therapeutic efficacy of Enco in mCRPC.
Keywords: BRAF, Encorafenib, invasion, metastatic castration-resistant prostate cancer, migration, PTEN

Özet: Metastatik kastrasyona-dirençli prostat kanseri (mKDPK), sınırlı tedavi seçeneği ve kötü prognozu olan oldukça agresif bir prostat kanseri (PKa) formudur. *BRAF* mutasyonları, nadir olmalarına rağmen, hücresel proliferasyon, hayatta kalma ve metastazda rol oynayan MAPK sinyal yolunu aktive ederek PKa'nın ilerlemesine katkıda bulunmaktadır. Bu çalışmada, farklı metastatik potansiyellere sahip mKDPK hücre hatlarında, ikinci nesil BRAF inhibitörü olan Encorafenib (Enco)'nin potansiyel antikanser ve anti-invaziv etkilerini orta düzeyde metastatik DU145 (*BRAF*-mutasyona uğramış) ve yüksek metastatik PC3 (*PTEN*-null) mKDPK hücre hatlarında ilk kez araştırdık. Sonuçlarımız, Enco'nun her iki hücre hattında da konsantrasyona ve zamana bağlı olarak hücre canlılığını azalttığını ve apoptozu indüklediğini göstermiş ve DU145 hücreleri ilaca daha duyarlı bulunmuştur. Enco, PC3 hücrelerinde migrasyonu inhibe ederken, DU145 hücrelerinde migrasyon üzerinde anlamlı bir etki göstermemiştir. Ayrıca, Enco tedavisi her iki hücre hattında da angiogenez ve invazyonla ilişkili genlerin (*VEGF-a*, *HIF1-a*, *MMP9* ve *MMP2*) ekspresyonunu arttırmıştır. Bu bulgular, Enco'nun mKDPK için sitotoksik bir ajan olarak potansiyel olabileceğini, ancak migrasyon, invazyon ve gen ekspresyonu üzerindeki etkilerinin kanser hücrelerinin spesifik genetik değişikliklerine bağlı olarak değişebileceğini düşündürmektedir. Bu durum, kişiselleştirilmiş tedavi stratejilerinin önemini ve adaptif direnç mekanizmalarının potansiyelini vurgulamaktadır. Enco'nun mKDPK'deki terapötik etkinliğini artırmak için özellikle birden fazla sinyal yolunu hedefleyen kombinasyon tedavilerine yönelik daha fazla çalışmaya ihtiyaç vardır.
Anahtar Kelimeler: BRAF, Encorafenib, invazyon, metastatik kastrasyona-dirençli prostat kanseri, migrasyon, PTEN

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1. Introduction

Prostate cancer (PCa) is a malignancy that predominantly affects middle-aged men and is the fifth leading cause of cancer-related mortality worldwide (1). The clinical management of PCa involves various approaches, including surgical intervention, chemotherapy, androgen deprivation therapy (ADT), and a range of advanced imaging modalities such as magnetic resonance imaging (MRI), transrectal ultrasound, computed tomography (CT), and positron emission tomography/computed tomography (PET/CT). Despite the progress in diagnostic and therapeutic strategies, the 5-year survival rate for patients with metastatic or advanced-stage PCa remains around 30% (2-4). While patients with advanced PCa initially respond to ADT, resistance to androgen deprivation and/or androgen receptor signaling blockade is a frequent and inevitable occurrence. The progression leads to the development of metastatic castration-resistant prostate cancer (mCRPC). This particularly aggressive and refractory disease phenotype resists most systemic therapies, resulting in a markedly poor prognosis and increased mortality (5, 6). Although various treatment options are available for mCRPC, including chemotherapy, endocrine therapy, immunotherapy, bone-targeted therapies, and poly-ADP-ribose polymerase (PARP) inhibitors, no curative treatment exists for mCRPC (7). Therefore, there is a pressing need for new therapeutic approaches for managing mCRPC.

The androgen receptor (AR) plays a critical role in the normal growth and development of the prostate gland and is pivotal in the pathogenesis of PCa (8). While the AR signaling pathway is central to PCa development, other genetic and molecular factors, including the MAPK signaling pathway and tumor suppressor gene *PTEN* mutations, contribute significantly to its progression (9). Additionally, activation of the RAS pathway has been identified in PCa (10). Mutations in the *BRAF* gene, a member of the RAF family, which regulates the MEK/ERK signaling pathway, significantly influence various aspects of tumor progression, including cellular proliferation, differentiation, migration, survival, and epithelial-mesenchymal transition (EMT). These mutations activate downstream signaling cascades that enhance oncogenic potential by promoting cell cycle progression, resistance to apoptosis, and increased invasiveness. Although *BRAF* gene mutations are relatively rare in PCa, occurring in approximately 3-5% of cases, they are associated with more aggressive disease phenotypes and may contribute to resistance against standard therapies, highlighting their clinical relevance in this subset of patients (11, 12).

All pathogenic *BRAF* mutations regulate transcription factors modulated via ERK phosphorylation, independent of the mutation type. This modulation is critical in driving gene expression changes that promote tumorigenesis, including cell proliferation, survival, and metastatic potential (13). *BRAF* mutations drive the expression of genes that initiate migration, invasion, and EMT through constitutive hyperactivation of survival and anti-apoptotic signaling pathways, such as MAPK, NF-κB, and PI3K/AKT, in various cancers. This dysregulated signaling promotes cellular plasticity and invasiveness and contributes to therapeutic resistance and metastatic progression (14). Therefore, it is hypothesized that BRAF-targeted therapy could reduce tumor aggressiveness by suppressing EMT-related gene expression.

In a study investigating the efficacy of Vemurafenib, a BRAF inhibitor, across various cancer types, including PCa, initial responses were observed in some patients. Still, the duration of these responses was notably short. Due to the limited number of PCa patients, definitive conclusions about Vemurafenib's effectiveness in this cancer type could not be drawn (15). Another study proposed inhibiting MEK downstream of BRAF could serve as a new treatment strategy in PCa with *BRAF* mutations (16). Consequently, BRAF-targeted therapies are promising approaches for treating PCa, particularly in cases with *BRAF* mutations.

Encorafenib (Enco), a second-generation BRAF inhibitor approved by the FDA for malign melanoma treatment, is a potent and highly selective ATP-competitive RAF inhibitor that targets $BRAF^{V600E}$, BRAF, and CRAF proteins (17). Although the anti-tumoral effects of Enco have been demonstrated in various cancers, such as colorectal cancer and melanoma (18, 19), its effect on migration and invasion abilities of mCRPC cells has yet to be investigated. Therefore, in the present study, we aim to investigate possible anticancer and anti-invasive effects of Enco in the mCRPC cells with high (PC3, *PTEN*-null) and moderate (DU145, *BRAF*-mutated) metastatic potentials for the first time.

2. Material and methods

2.1. Cell culture conditions and MTT assay

Enco (99.81% purity, LGX818) was purchased from Selleck Chemicals (Houston, TX, USA) and prepared in 0.01% dimethyl sulfoxide (DMSO) (1mM stock solution) (Santa Cruz Biotechnology, Dallas, TX, USA) solution. The DU145 (HTB-81TM) and PC3 (CRL-1435™) mCRPC cells were purchased from the American Type Culture Collection (ATCC). mCRPC cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing L-glutamine and supplemented with 1% sodium pyruvate, 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 1% antibiotic-antimycotic solution (Capricorn Scientific, Germany). The mCRPC cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2-3 days; then, the cells were passaged using Trypsin EDTA 0.25% (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at the 80% density for the following experiments. Briefly, mCRPC cells were seeded into 96-well plates (1.5×10^4 cells/well) overnight in a 100 µl medium, and the concentrations of the Enco were selected as 1, 10, 25, 50, 75, 100, 250, 500, 750, and 1000 nM respectively, based on the previous studies (20, 21).

Subsequently, the cytotoxic effect of Enco for the indicated concentrations at 24, 48, and 72 h was assessed using the MTT (Thiazolyl blue tetrazolium bromide) (Sigma-Aldrich, St Louis, MO, USA) assay. MTT dye was prepared in PBS in a 5 mg/mL solution (22). The cells were treated with Enco for 24, 48, and 72 h. 10 µl MTT reagent was added to each well and incubated for 3 h at 37°C and 5% CO_2. After incubation, 100 µl DMSO was added to each well to dissolve formazan crystals. Then, cell viability was detected in triplication by measuring absorbances at 570 nm using a TriStar2 LB 942 monochromator microplate reader (Berthold Technologies, Bad Wildbad, Germany).

2.2. Apoptotic assay

Based on the MTT assay, mCRPC cells were cultured into 6-well plates (1×10^5 cells/well) overnight, and the cells were treated with 750 and 1000 nM Enco for 72h. After incubation, the cells were harvested and washed with PBS. The staining protocol was performed with a Muse Annexin V & Dead Cell Kit (Merck Millipore, Germany). Apoptotic cells were analyzed in three replicates using a Muse Cell Analyzer (Merck Millipore, Germany).

2.3. Scratch assay

A scratch assay was performed to evaluate the effect of Enco on the migration ability of mCRPC cells. The cells were cultured into 6-well plates (1×10^6 cells/well) overnight. After incubation, scratches were created using a 200 µl pipette tip, washed with PBS, and the cells were treated with 750 and 1000 nM Enco for 72 h. The cells were washed once with PBS and observed under an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) at 0 and 72 h. At least 6 different images were obtained from each experiment group. The migratory ability of Enco-treated cells compared to the non-treated control group was analyzed using the Image*J* program (National Institutes of Health, Bethesda, MD, USA).

2.4. Gene expression analysis

We used real-time quantitative polymerase chain reaction (RT-qPCR) analysis to determine the regulatory effect of Enco on angiogenesis and invasion-related gene expression levels in mCRPC cells. The cells were cultured into 6-well plates (1×10^6 cells/well) overnight and treated with 750 and 1000 nM of Enco for 72 h. Then, total RNA extraction was performed using GeneALL Hybrid R (GeneAll Biotechnology, Seoul, Korea). All RNA quality was checked using a spectrophotometer (Beckman Coulter, USA) by measurement of absorbance at 260/280 nm. Firstly, 150 ng of each RNA sample was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Then, RT-qPCR was performed for *VEGF-a*, *HIF1-a*, *MMP9*, and *MMP2* genes. The *GAPDH* gene expression level was used for normalization, and all analysis was performed in triplicate using A.B.T.TM 2X qPCR SYBR-Green Master Mix (Ankara, Turkiye) protocol on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). After determining the C*t* values, statistical analysis was performed using a web-based tool at https://www.qiagen.com/us/applications/geneglobe/qpcr-data-analysis.

2.5. Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0 (La Jolla, CA). All results were expressed as the mean ± standard deviation (SD). The difference between treated and control groups was

determined using an analysis of variance (ANOVA) with an appropriate post-hoc test. If the $p<0.05$, statistical significance was accepted in all comparisons.

3. Results

We have shown that Enco treatment caused a significant reduction in the viability of DU145 and PC3 mCRPC cells in a time- and concentration-dependent manner. Cell viability results indicated Enco has significant cytotoxic effects (nearly 50%) on mCRPC cells at concentrations above 500 nM for 72 h. As shown in Figure 1A, Enco reduced the viability of DU145 cells to 63.7%, 61.0%, and 53.6% at 500, 750, and 1000 nM, respectively, after 72 h ($p<0.01$). However, under the same treatment conditions, the viability of PC3 cells decreased by 63.5%, 61.5%, and 61.3% ($p<0.01$) (Figure 1B). Thus, based on the cell viability results, we showed that Enco exhibited more cytotoxic effects at maximum concentration (1000 nM) on DU145 cells than on PC3 cells for the most effective time at 72 h.

When the apoptotic effect of Enco on mCRPC cells was compared, we found that Enco caused a slight increase in apoptotic cell death, rising from 13.1% to 17.2% and 25.5% at the 750 nM and 1000 nM treatment groups in DU145 cells after 72 h, respectively ($p<0.01$) (Figure 1C). However, in PC3 cells, the total apoptotic rate increased from 12.8% to 19.3% and 17.9% after 750 nM and 1000 nM Enco treatments for 72 h, respectively ($p<0.01$) (Figure 1D). The statistical results of apoptosis are shown in Figure 1E. Thus, we suggest that Enco, a potent autophagy inducer, has a more apoptotic effect on DU145 cells than on PC3 cells.

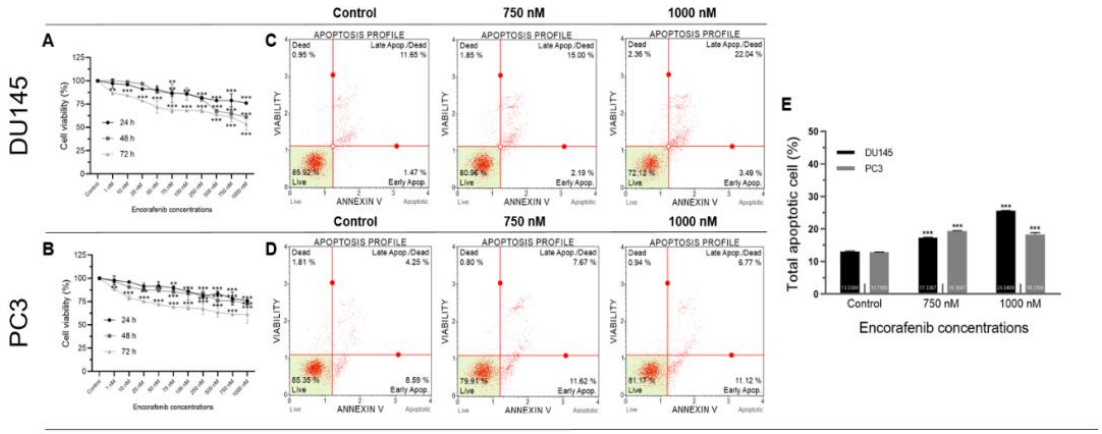

Figure 1. *Cytotoxic effect of Enco at increasing concentrations on (A) DU145 and (B) PC3 mCRPC cells for 24, 48, and 72 h (Each treated group was compared separately with the non-treated control group, *p<0.05, **, ***p<0.01). Apoptotic effect of Enco at 500, 750, and 1000 nM on (C) DU145 and (D) PC3 cells for 72 h. (E) Statistical results of the apoptotic assay at increasing concentrations of Enco compared to the non-treated control group for 72 h (***p<0.01).*

We compared the effect of Enco on the migration capacity of mCRPC cells. The scratch assay results showed that Enco differentially altered the migration ability of mCRPC cells at 750 nM and 1000 nM treatments for 72 h (Figure 2). No significant changes were detected in the migration ability of Enco-treated DU145 cells, as shown in Figure 2A. However, Enco caused a significant decrease in the migration of PC3 cells (Figure 2B). Unlike DU145 cells, Enco exhibited a significant anti-migratory effect on PC3 cells at the same treatment conditions ($p<0.01$) (Figure 2B). This effect was more pronounced in the 500 nM treatment group compared to the 1000 nM group. Therefore, we concluded that while Enco has no anti-migratory effect on *BRAF*-mutated DU145 mCRPC cells, a variable, non-concentration-dependent effect was observed in *PTEN*-null PC3 mCRPC cells. The statistical representation of the results is also shown in Figure 2C.

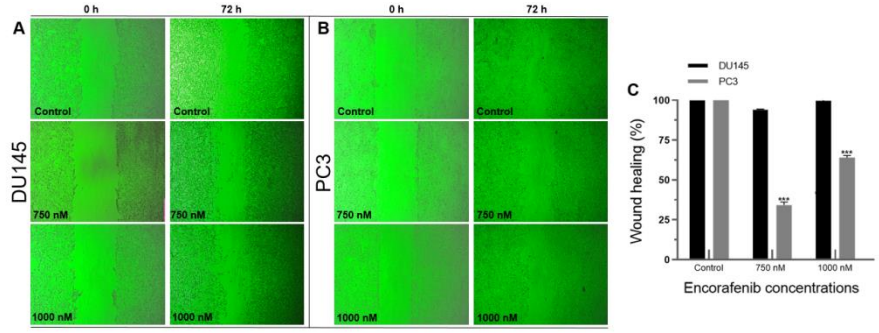

Figure 2. *Anti-migratory effect of Enco on (A) DU145 and (B) PC3 mCRPC cells at 750 and 1000 nM for 0 h and 72 h. (C) Graphical representation of scratch assay results of each treated group was compared separately with the non-treated control group at 72 h (***p<0.01).*

Next, we analyzed the gene expression levels related to angiogenesis, invasion, and migration in Enco-treated DU145 and PC3 mCRPC cells. Despite its cytotoxic effect, gene expression results indicated that Enco treatment increased the mRNA levels of *VEGF-a*, *HIF1-a*, *MMP9*, and *MMP2*. In DU145 cells, Enco treatments caused a significant 6.21-fold increase (p<0.05) in *VEGF-a* expression at 750 nM after 72 h. Moreover, the expression levels of *VEGF-a*, *HIF1-a*, and *MMP9* increased by 5.32-fold (p<0.01), 3.15-fold (p<0.01), and 2.52-fold (p<0.05) compared to the non-treated control group, respectively (Figure 3A). In PC3 cells, 750 nM Enco treatment increased the gene expression levels of *VEGF-a*, *HIF1-a*, *MMP9*, and *MMP2* to 6.72-fold (p<0.01), 3.89-fold (p<0.01), 4.82-fold (p<0.01), and 4.17-fold (p<0.01), respectively. Finally, 1000 nM Enco treatment for 72 h also increased the expression levels of *VEGF-a*, *HIF1-a*, *MMP9*, and *MMP2* by 2.63-fold (p<0.05), 2.46-fold (p<0.05), 1.66-fold (p<0.05), and 1.89-fold (p<0.05) compared to the non-treated control group, respectively (Figure 3B).

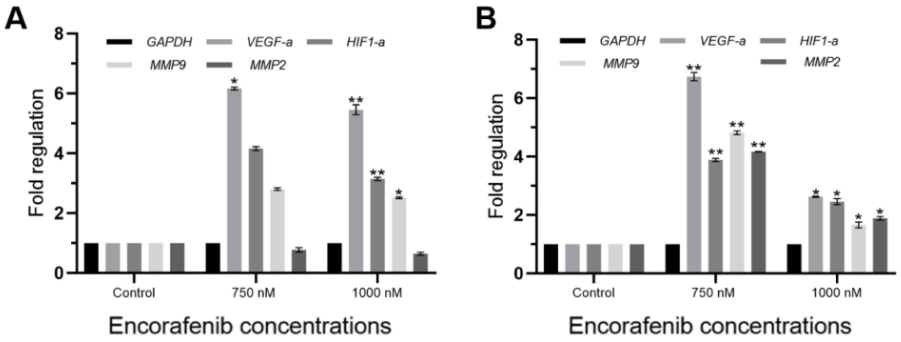

Figure 3. *The effect of Enco on the expression levels of VEGF-a, HIF1-a, MMP9, and MMP2 genes in (A) DU145 and (B) PC3 mCRPC cells after treatments with 750 and 1000 nM for 72 h (Each treated group was compared separately with the non-treated control group for each gene expression, *p<0.05, **p<0.01).*

4. Discussion

Metastatic castration-resistant prostate cancer (mCRPC) remains a significant clinical challenge in treatment (5-7). In the current study, we investigated the potential of Enco, a second-generation BRAF inhibitor, to affect migration and invasion ability in mCRPC cells. Our findings showed that while Enco significantly inhibited cell viability and induced apoptosis, it had a variable effect on migration and increased invasion properties of high metastatic *PTEN*-null PC3 and moderate metastatic *BRAF*-mutated DU145 cells.

BRAF mutations play a crucial role in the progression of PCa, although their prevalence is low (10). These mutations are typically associated with more aggressive clinical features in patients. All pathogenic *BRAF* mutations influence transcription factors via ERK phosphorylation, irrespective of the specific mutation. This alteration is essential for modifying gene expression and promoting tumorigenesis through cell proliferation, survival, and metastasis. *BRAF* mutations activate several signaling pathways, such as MAPK, NF-κB, and

PI3K/AKT, which upregulate genes involved in migration, invasion, and EMT (23). This aberrant signaling enhances cellular plasticity and invasiveness, facilitating therapeutic resistance and metastasis progression. As a result, BRAF-targeted therapies are hypothesized to mitigate tumor aggressiveness by inhibiting the expression of genes related to EMT (24).

Vemurafenib, a BRAF inhibitor, has been evaluated for its effectiveness across various cancer types, including prostate cancer (PCa). While some patients showed initial positive responses, the duration of these responses was notably brief. Given the limited number of PCa patients in the studies, definitive conclusions regarding Vemurafenib's efficacy in this cancer type remain elusive (15). Another investigation proposed that targeting MEK, a downstream mediator of BRAF, might be a promising treatment strategy for PCa patients with *BRAF* mutations (16). Consequently, BRAF-targeted therapies are considered a potentially valuable approach for treating PCa, particularly in cases with BRAF mutations. Enco, a second-generation BRAF inhibitor, is a potent and highly selective ATP-competitive RAF inhibitor compared to other BRAF inhibitors (17, 25). While Enco has demonstrated anti-tumor effects in various cancers, such as colorectal cancer and melanoma (18, 19), its influence on the migration and invasion abilities of mCRPC cells has not been explored.

In the current study, we investigated possible anticancer and anti-invasive effects of Enco in the mCRPC cells with high (PC3, *PTEN*-null) and moderate (DU145, *BRAF*-mutated) metastatic potentials for the first time. Then, the results suggested that Enco has significant cytotoxic and apoptotic effects on DU145 and PC3 mCRPC cells above 500 nM for 72 h. However, these effects were more prominent in *BRAF*-mutated DU145 cells, which have a moderate metastatic potential compared to *PTEN*-null PC3 cells with high metastatic potential. Enco also caused differential anti-migratory effects on the cells. While Enco has no changeable anti-migratory effect, the migration ability of PC3 cells was significantly inhibited after Enco treatments. However, despite this effect, Enco treatment significantly increased expression levels of the genes related to angiogenesis, invasion, and migration in both cells. Thus, we concluded that Enco differentially alters the invasion ability of mCRPC cells based on variable genetic and metastatic features of the cell lines.

The differential effects observed in cell viability and apoptosis could be attributed to these two cell lines' distinct molecular genetic alterations. DU145 cells harbor a *BRAF* mutation, which leads to constitutive activation of the MAPK/ERK signaling pathway, a key driver of tumor progression and metastasis. The heightened sensitivity of DU145 cells to Enco may be related to the inhibition of this pathway, as BRAF inhibitors like Enco are known to target the aberrant BRAF signaling in mutated cells. In contrast, PC3 cells lack PTEN expression, which results in constitutive activation of the PI3K/AKT pathway, another major signaling axis involved in cell survival, migration, and invasiveness (26, 27). While *PTEN*-null cells generally exhibit more aggressive behavior, the reduced sensitivity to Enco in PC3 cells suggests that the PI3K/AKT pathway may not be as directly impacted by BRAF inhibition, limiting the efficacy of Enco in these cells.

Interestingly, while Enco significantly inhibited the migration ability of PC3 cells, it did not alter the migratory properties of DU145 cells. This finding suggests that Enco's effects on migration might be context-dependent and influenced by the specific molecular features of each cell line. The observed inhibition of migration in PC3 cells may be related to the broader impact of Enco on the PI3K/AKT pathway, which plays a critical role in cellular motility (28). In contrast, DU145 cells may rely on alternative mechanisms for migration, such as the BRAF/MEK/ERK pathway, which might not be as profoundly affected by Enco in the same conditions (29).

A particularly intriguing observation was an increase in the expression of genes related to angiogenesis, invasion, and migration following Enco treatment in both cell lines. This paradoxical effect suggests that, despite inhibiting migration in PC3 cells, Enco may promote the expression of genes associated with tumor progression, potentially through compensatory mechanisms or feedback loops. Such findings raise important questions about the potential for BRAF inhibition to induce adaptive resistance mechanisms, which could ultimately limit the long-term effectiveness of Enco as a monotherapy. This phenomenon has been observed in other cancers where targeted therapies initially induce promising results, but tumors evolve to activate alternative pathways that promote metastasis and drug resistance (30).

These results underscore the complexity of targeting molecular pathways in cancer cells with

heterogeneous genetic backgrounds. As a limitation of the study, the effects of Enco were not evaluated in any normal cells since Enco is an approved drug for MM patients. However, in addition to the cytotoxic and apoptotic effects of the drug, Enco's differential effects on invasion and migration in PC3 and DU145 cells highlight the need for personalized approaches in mCRPC treatment, as the therapeutic outcomes of BRAF inhibition may depend heavily on the specific genetic and molecular characteristics of the tumor. Furthermore, the upregulation of migration, invasion, and angiogenesis-related genes suggests that combination therapies targeting multiple signaling pathways, including the PI3K/AKT and MAPK/ERK pathways, may be necessary to achieve more sustained therapeutic efficacy in mCRPC (31, 32).

5. Conclusion

In conclusion, our study provides valuable insights into the varying effects of Enco on mCRPC cells with different metastatic potentials. This differential response between the two cell lines suggests that Enco's therapeutic efficacy may vary depending on the genetic profile and metastatic characteristics of the cancer cells. While Enco shows promise in inhibiting migration in PC3 cells, the increase in invasion and migration-related gene expression emphasizes the complexity of its action and the potential for adaptive resistance mechanisms. Future studies should explore combination therapies to overcome these limitations and further elucidate the role of Encorafenib in mCRPC treatment.

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