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PREFACE

Cancer continues to be one of today's major health problems. In particular, prostate cancer is one of the most common cancers in men and when it reaches the metastatic stage, treatment options are quite limited. Therefore, research into new treatment approaches and effective molecules are becoming increasingly important.

In this book, the molecule of 3,5-bis(2-fluorobenzylidene)-4-piperidone (EF24), as an analog of the main component of turmeric (Curcuma longa), was developed to address the low bioavailability of curcumin and was studied by applying it to DU145 and Pc3 prostate cancer cell lines. The cytotoxic and metastasis inhibitory effects of EF24 were investigated. The findings suggest that naturally derived molecules may be promising alternatives for the treatment of prostate cancer in the future.

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THE EFFECT OF 3,5-BIS(2-FLUOROBENZYLIDENE)-4-PIPERIDONE (EF24) MOLECULE ON THE METASTASIS MECHANISM IN PROSTATE CANCER CELLS

Abstract—Prostate cancer is one of the most common types of cancer in men. Current therapies impair patients' quality of life and are often inadequate. Therefore, developing alternative therapies and new drugs has become increasingly important. This study investigated the effect of the curcumin analog EF-24 on prostate cancer cells. A comprehensive cytotoxicity analysis was performed with PNT1A normal prostate cells and DU145 and PC3, two different metastatic prostate cancer cell lines. In addition, a two-dimensional migration assay and qRT-PCR method were used to identify the impact of EF-24 on the metastatic mechanism of prostate cancer. EF-24 was found to be specifically cytotoxic to prostate cancer cells at low doses. In addition, EF-24 inhibits the metastatic mechanism, probably by increasing E-cadherin. Natural components are coming to the fore in cancer drug research. Curcumin has been used in various fields, and its effect on various cancers has been shown in vitro and in vivo studies. Due to the low bioavailability of curcumin, various analogs have been developed and EF-24 is one of the best known of these analogs. Its effect on various types of cancer has been studied and it is considered a potential drug. The results we obtained are consistent with those found in the

literature. In conclusion, EF-24 inhibits the proliferation and migration of prostate cancer cells. Although the results obtained in our study show the potential of EF-24 in the therapy of prostate cancer, it needs to be supported by extensive molecular research and in vivo experiments.

Key words: curcumin analog, EF-24, prostate cancer, metastasis, DU145, PC3, PNT1A.

INTRODUCTION

According to the International Agency for Research on Cancer (IARC), the number of newly detected cancers worldwide will reach 19 million in 2020, while the number of cancer-related deaths will be around 10 million. Prostate cancer patients account for 7% of newly diagnosed cancer patients and about 4% of cancer deaths. Prostate cancer is the most common type of cancer in men (Bergengren, et al. 2023).

In addition to the conventional treatments used to treat cancer, research into alternative therapies has increased in recent years. Recent studies have shown that chemicals derived from natural substances have chemopreventive and therapeutic effects (Yang, et al. 2014). The best example of this is curcumin, the main component of turmeric in cancer treatment. In addition to its use as a spice in Asian cuisine, turmeric has proven ancient medicinal applications and has been used as an analgesic and antibiotic to accelerate wound healing (Supplementary Figure S1; Supplementary Figure S2- Additional background information can be found in Supplementary File 1.) (Aggarwal, et al. 2007). On the other hand, curcumin, which is in phase I clinical trials, is safe even at high doses (12 g/day) in humans. Still, its poor bioavailability limits the use of curcumin in cancer treatment (Cheng, et al. 2001). Curcumin's poor absorption by the gut and rapid elimination from the body has led to the development of more effective analogs (Anand, et al. 2007). Brian K. Adams and his team initiated the first development of curcumin analogs in 2004. The best-known of the analogs synthesized and tested for anti-cancer activity was EF-24, also known as 3,5-bis(2-fluorobenzylidine)-4-piperidone (Adams, et al. 2004). Like curcumin, EF-24 is an α,β-unsaturated ketone and an electrophile, that can react with the sulfhydryl group (ZSH) on intracellular molecules such as GSH and thioredoxin (Supplementary Figure S5) (Shoji, et al. 2008). EF24 was shown to have promising bioactivities, especially its anti-cancer activity in various solid tumors and leukemia (He, et al. 2018). In addition, the EF-24 molecule was shown in this study to be a more effective chemotherapeutic agent with fewer side effects than cisplatin, which is used for most cancers, even at high doses in mice. Since the work of Brian K. Adams et al, EF-24 has been tested on numerous cancer cell lines. The effect of the EF-24 molecule on prostate cancer has been examined in three studies (Yang, et al. 2013; Adams, et al. 2005; Thomas, et al. 2008). The findings demonstrated that the EF-24 molecule reduced proliferation, induced apoptosis, and inhibited cancer metabolism in prostate cancer cell lines. In addition, Yang et al. showed in vivo experiments on mice that the tumor formed with DU145 cells decreased in size by 65% after treatment with EF-24.

The effect of the EF-24 molecule on metastasis was investigated in melanoma, cholangiocarcinoma, hepatocellular, and ovarian cancer.

In cholangiocarcinoma, EF-24 has been shown to inhibit the NF-κB signaling pathway, decrease N-cadherin and vimentin, and increase E-cadherin levels (Yin, et al. 2016). In ovarian cancer, EF-24 has been shown to inhibit metastasis via the GLUT1 protein (Zhang, et al. 2013). In melanoma, EF-24 has been indicated to inhibit the NF-κB signaling pathway, decrease N-cadherin, vimentin, and HMGA2 levels, and increase E-cadherin levels (Yang, et al. 2013; Zhang, et al. 2015). Finally, EF24 has been shown to inhibit metastasis in hepatocellular cancer by reducing Src protein phosphorylation, and EF24 has been shown to inhibit filopodia (invadopodia) formation (Zhao, et al. 2016).

Metastasis is the blood migration of cancer cells after epithelial-mesenchymal transition (EMT) from the site of origin to another site and their growth at this site (Sever & Brugge, 2015). During EMT, epithelial cells decrease the expression of E-cadherin and start to express N-cadherin (Derynck & Weinberg, 2019). There are numerous oncogenic signaling pathways associated with cadherin cell-cell adhesion. Thus, N-cadherin activates the PI3K pathway by stabilizing the FGF receptor, MMP-9 expression is increased (Suyama, et al. 2002). Since most solid tumors in humans are of epithelial origin, adhesion molecules and cell signaling pathways at the attachment sites of epithelial cells are of great interest. Recent insights into the roles of E- and N-cadherins in cancer invasion and metastasis, along with their crosstalk with other signaling pathways, have emerged. Most invasive and metastatic cancers are correlated with high levels of E-cadherin expression, especially in people with prostate cancer (Putzke, et al. 2011). E-cadherin-mediated adhesion linkages suppress stimulation of the Wnt/β-catenin pathway and RTK/P13K pathway in epithelial cells. N-cadherin-mediated adhesion compounds together with platelet-derived growth factor receptor (PDGFR) facilitate the activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) and phosphoinositol 3-kinase (PI3K) and increase cell survival and migration in non-epithelial cells (Loh, et al. 2019).

This study examined the cytotoxic effect of the EF-24 molecule on the prostate cancer cell lines DU145 and PC3. Compared to existing literature, normal PNT1A prostate cells were utilized to identify the dose that exhibited a specific cytotoxic effect on prostate cancer cells while sparing normal cells. To achieve this, MTT assays were conducted across a concentration range of 0.625 to 20 µM with 24, 48, and 72-hour incubation periods. The capacity of EF-24 to inhibit the metastasis of prostate cancer cells was assessed using scratch assay and qRT-PCR techniques.

The expression levels of E-cadherin, N-cadherin, vimentin, MMP-9, and Twist-1 genes were analyzed through the qRT-PCR method. These adhesion molecules are new, promising targets in cancer therapy because cadherins show diverse biological functions. High N-cadherin expression is associated with tumor aggressiveness, cancer metastasis, apoptosis, and angiogenesis. Because of this purpose, this current research EF-24 may also be a good diagnostic tool for prostate cancer treatment.

MATERIALS AND METHODS

Cell culture. PC3 cells were kindly provided by Merve Sezer Kurkcu (Mugla University), DU145 cells by Fatih Kocabas (Yeditepe University), and PNT1A cells by Omer Faruk Karatas (Erzurum Technical University). PC3 and DU145 cells were grown in high glucose DMEM (Sigma-D6429-500ML) containing 10% fetal bovine serum (Biowest, S181H-500), 1% L-glutamine (Gibco, 25030081) and 1% penicillin-streptomycin (10,000 U/mL-10,000 μg/mL) (Gibco, 10378016). PNT1A cells were grown in RPMI 1640 (Gibco, 21875034) with 10% fetal bovine serum (Biowest, S181H-500), 1% L-glutamine (Gibco, 25030081), 1% penicillin-streptomycin (10,000 U/mL-10,000 μg/mL) (Gibco, 10378016). Cell culture was performed in an incubator with 5% CO_2 and 37°C.

Cell viability/cytotoxicity test (MTT). The MTT assay was performed to investigate the cytotoxic effect of EF-24 on cancer and normal cells. For this assay, PC3, DU145 and PNT1A cells were cultured and plated in 96-well plates. PC3 and DU145 cells were seeded at 3000 cells per well, while PNT1A cells were plated at 4000 cells per well.

After one day (24 hours), the medium of the cells was replaced with culture media containing different concentrations of EF-24. Docetaxel was used as a reference drug and as a positive control. DMSO was used to dissolve EF-24 and docetaxel at a concentration of 0.1% was used as a negative control. To prevent the degradation of EF-24 from the culture media, the medium of the cells to be tested with

MTT after 48 hours was changed for 24 hours. Similarly, the media for the cells to be subjected to MTT after 72 hours was replaced after 24 and 48 hours with media containing EF-24, docetaxel or DMSO. The MTT assay (Biotium, 30006) was applied at 24, 48, and 72 hours according to the manufacturer's instructions. Briefly, 10 µL of MTT solution was added to each well containing 100 µL of culture medium, mixed gently, and incubated for 4 h in an incubator at 37°C and 5% CO2. After this time, 200 µL DMSO was added and pipetted until the formazan salts had dissolved. The absorbance was measured using a microplate reader (Tecan Infinite 200pro). The measurements were taken at 570 nm and 630 nm. The absorbance measured at 630 nm was used to detect the background signal.

Scratch/wound healing test. The scratch/wound healing test was performed to measure the migration ability of the cells. For this experiment, the cells were seeded in a 24-well plate. One day later, after removing the medium, a black line was drawn with a marker on the bottom of the plate, which served as a reference for imaging. A scratch was made in the center of the well with a 200-µl tip. To remove the screened cells, media with different concentrations of EF-24 or 0.1% DMSO were added after washing with PBS. Media with 5% FBS measured only migration without inhibiting proliferation. Images were taken after 0 and 8 hours under a Differential Interference Contrast (DIC) microscope and analyzed with Scratch software.

qRT-PCR. A qRT-PCR assay was performed to measure the expression of genes related to the metastatic pathway. DU145, PC3 and PNT1A cells were seeded in 6-well plates and treated with EF-24 or

0.1% DMSO 24 hours later. After 72 hours, each well was removed separately with Magzol from the gastric RNA isolation kit. RNA isolation was performed according to the kit protocol and stored at -20°C in nuclease-free water. After measuring the amount of RNA, cDNA synthesis was performed (A.B.T cDNA synthesis kit). With the obtained cDNAs, qPCR was performed for different genes. The sequences of the primers used are listed in Table 1. GADPH was used as housekeeping gene. The PCR conditions were 95°C for 300 seconds, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Table 1: Primer sequences used for qRT-PCR experiments

Gene	Forward primer	Reverse primer
Vimentin	TAACCAACGACAAA GCCCGC	TGCAGGGTGTTTTCGG CTTC
E-cadherin	CTGGTGGTTCAAGCT GCTGAC	ACCTGACCCTTGTACG TGGTG
MMP-9	AGTCCACCCTTGTGC TCTTCC	TGCCACCCGAGTGTAA CCAT
N-cadherin	CCAGAAAACTCCAGG GGACCT	CCCAGTTACACGTATC CGCAG
Twist1	CCATGTCCGCGTCCC ACTA	CCCACGCCCTGTTTCTT TGAAT
GADPH	CTTCTTTTGCGTCGCC AGCC	TCCGTTGACTCCGACC TTCAC

Statistical analysis. GraphPad Prism 9 software was used to analyze the OD values obtained in the MTT experiment. Six replicates were performed for each concentration, and the controls were tested in the MTT experiment. All experimental groups were compared with the data that passed the Shapiro-Wilk and Kolmogorov-Smirnov normality test and were compared with Tukey's significance test and one-way analysis of variance tests. $p<0.05$ results were considered significant.

Three replicates of each sample were performed in the scratch/wound healing experiment. The results obtained were analyzed using Scratch software, and the relative wound closure data was analyzed using GraphPad Prism 9 software. Data that passed the Shapiro-Wilk and Kolmogorov-Smirnov normality tests were compared using the t-test. Results with $p<0.05$ were considered significant.

The qRT-PCR experiments were set up as three biological and three technical replicates. The Ct values obtained were analyzed using the Livak method and evaluated with GraphPad Prism 9 software. Data that passed the Shapiro-Wilk and Kolmogorov-Smirnov normality tests were compared with Dunnett's test and one-way analysis of variance tests. Results with $p<0.05$ were considered significant.

RESULTS

EF-24, DU145, and PC3 have a cytotoxic effect on human prostate cancer cells, whereas they do not affect normal PNT1A prostate cells.

The MTT assay is commonly used to measure cytotoxicity. In our experiment, different doses of EF-24 were tested. To perform a synthetic study, MTT assays were performed at 24, 48 and 72 hours. Only comparisons with the 0.1% DMSO control are shown in the graphs. Docetaxel was utilized as a positive control. In our cytotoxicity assay, we used twice the IC50 for DU145 and PC3 as described in the literature (Yang, et al. 2019).

As shown in Figure 1A, the doses of 0.625 µM, 1.25 µM, 2.5 µM and 5 µM EF-24 applied to normal PNT1A prostate cells for 24 hours had no significant cytotoxic effect compared to the 0.1% DMSO control. In contrast, 10 µM and 20 µM EF-24 doses caused significant cytotoxicity compared to the control. On the other hand, no cytotoxicity was observed at 0.625 µM, 1.25 µM and 2.5 µM EF-24 doses in the 48-hour assay, while a cytotoxic effect of up to 50% was observed at 5 µM, 10 µM and 20 µM EF-24 doses (Figure 1B). Finally, after 72 hours, no cytotoxic effect was observed at 0.625 µM, 1.25 µM and 2.5 µM EF-24, while a significant cytotoxic effect was observed at 5 µM, 10 µM and 20 µM EF-24 (Figure 1C). 8 nM docetaxel, used as a control, caused no significant cytotoxic effect in normal PNT1A prostate cells at 24, 48 and 72 hours compared to the 0.1% DMSO control.

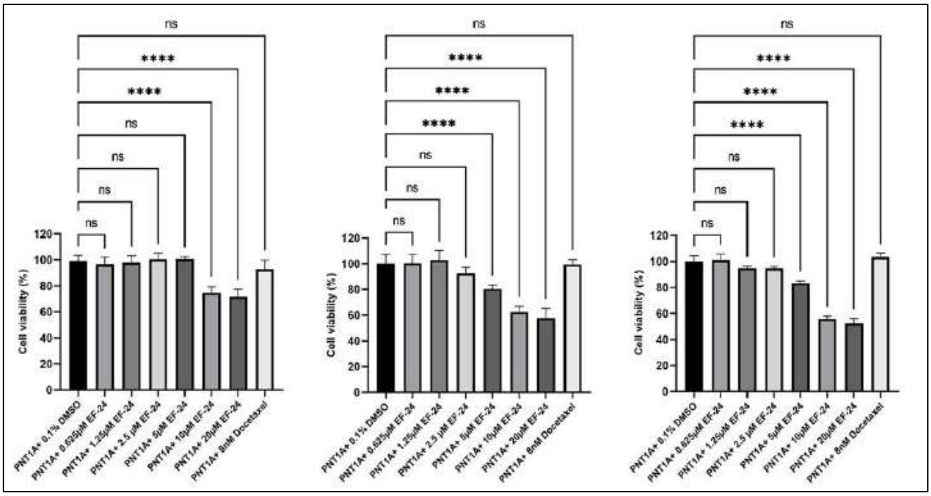

Figure 1. Cytotoxic effect of EF-24 molecule on PNT1A normal prostate cells. A)24 hours B)48 hours C)72 hours (*p <0.05).

As shown in Figure 2, the EF-24 molecule induces a cytotoxic effect on DU145 prostate cancer cells dose-dependently at 24, 48, and 72 hours. Except for the 0.625 µM EF-24 dose at 24 hours, 1.25 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM EF-24 doses had a significant cytotoxic effect on DU145 cells compared to the 0.1% DMSO control at 24 hours, 48 hours and 72 hours. In addition, no significant difference was measured between 2.5 µM and 5 µM EF-24 doses at 24, 48and 72 hours (not shown in the graph). 8 nM docetaxel, used as a control, produced significant cytotoxic effects in DU145 prostate cancer cells at 24, 48, and 72 hours compared to the 0.1% DMSO control. There was no significant difference between the cytotoxic effect measured with docetaxel and the 2.5 µM EF-24 dose (not shown in the graph).

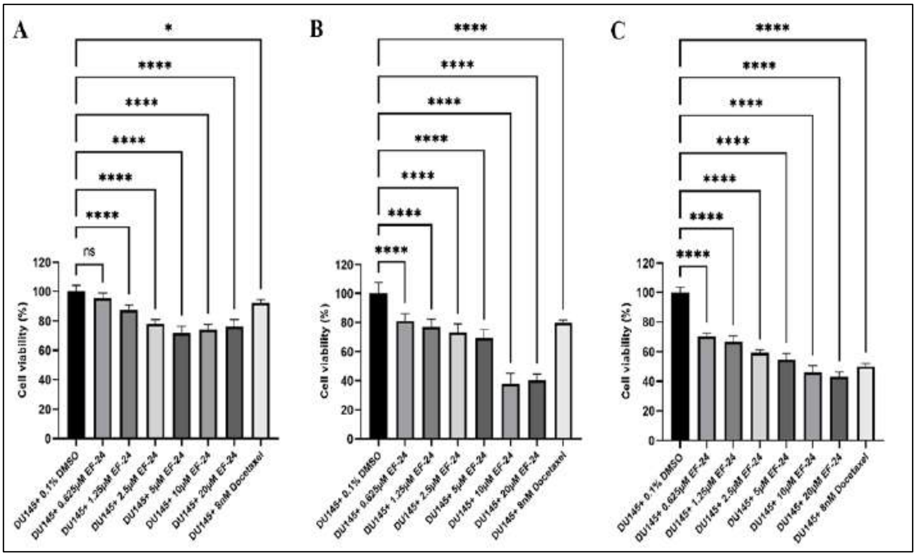

Figure 2. Cytotoxic effect of EF-24 molecule on DU145 prostate cancer cells. A)24 hours B)48 hours C)72 hours (*p <0.05).

As can be seen in Figure 3, except the 0.625 µM EF-24 dose, 1.25 µM, 2.5 µM, 5 µM, 10 µM and 20 µM EF-24 doses produced a significant cytotoxic effect on PC3 cells at 24, 48and 72 hours compared to the 0.1% DMSO control. This cytotoxic effect increased in a dose-dependent manner. Furthermore, no significant difference was measured between 2.5 µM and 5 µM EF-24 doses after 24, 48and 72 hours (not shown in the graph). 8 nM docetaxel, used as a control, induced significant cytotoxic effects in DU145 prostate cancer cells at 48 and 72 hours compared to the 0.1% DMSO control. The cytotoxic effect measured with docetaxel was significantly lower than that measured with the 2.5 µM EF-24 dose (not shown).

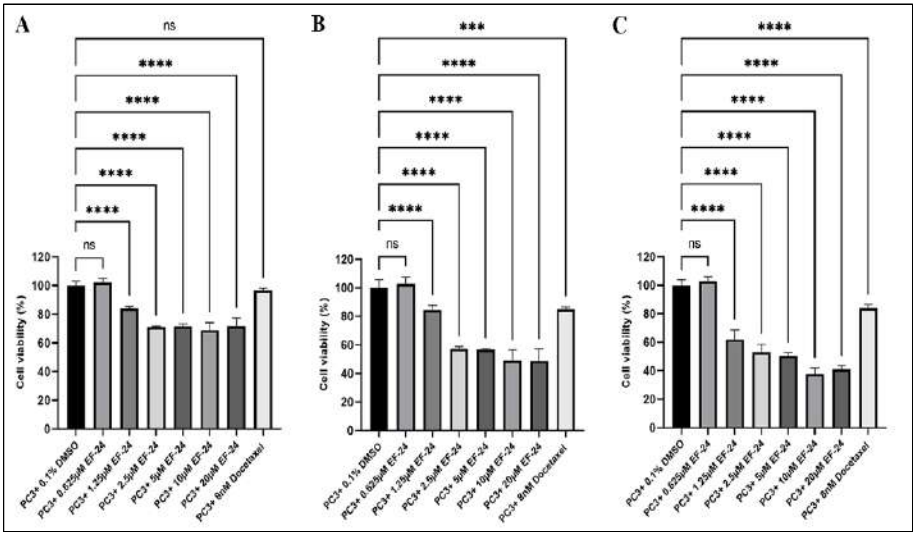

Figure 3. Cytotoxic effect of EF-24 molecule on PC3 prostate cancer cells. A)24 hours B)48 hours C)72 hours (*p <0.05).

EF-24 potently inhibits metastasis of DU145 and PC3 human prostate cancer cells. Wound healing assays and qRT-PCR experiments were performed to investigate the effect of the EF-24 molecule on cancer metastasis. While a two-dimensional investigation was carried out in the wound healing experiment, the qRT-PCR experiment attempted to uncover the molecular mechanism.

In the wound healing experiment, an artificial wound was created in confluent cells and the migration capacity of the cells was measured. Each condition was repeated 3 times and the statistical analysis was performed. As shown in Figure 4, when 2.5 µM EF-24 was applied to the DU145 prostate cancer cell lines, the wound formed was significantly less closed after 8 hours compared to the DMSO control. Figure 5 shows that there was no significant difference in wound closure when 2.5 µM EF-24 was applied to PC3 prostate cancer cells compared to the DMSO control.

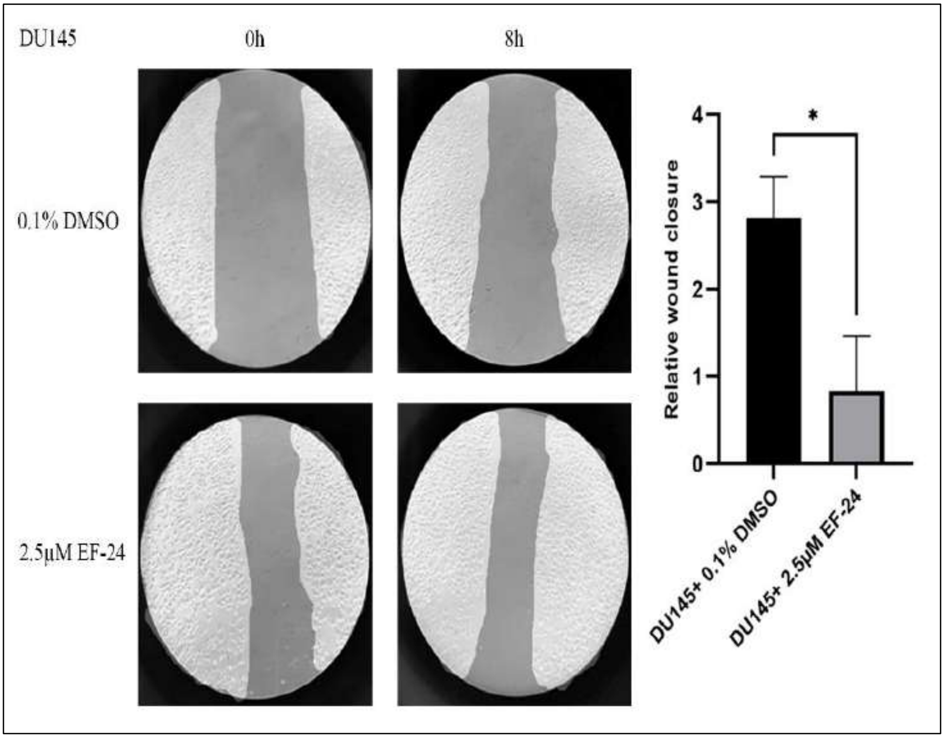

Figure 4. Measuring the effect of EF-24 on migration of DU145 cancer cell lines by wound healing assay. Right: DIC images after Scratch analysis. Left: Statistical analysis of data obtained after Scratch analysis.

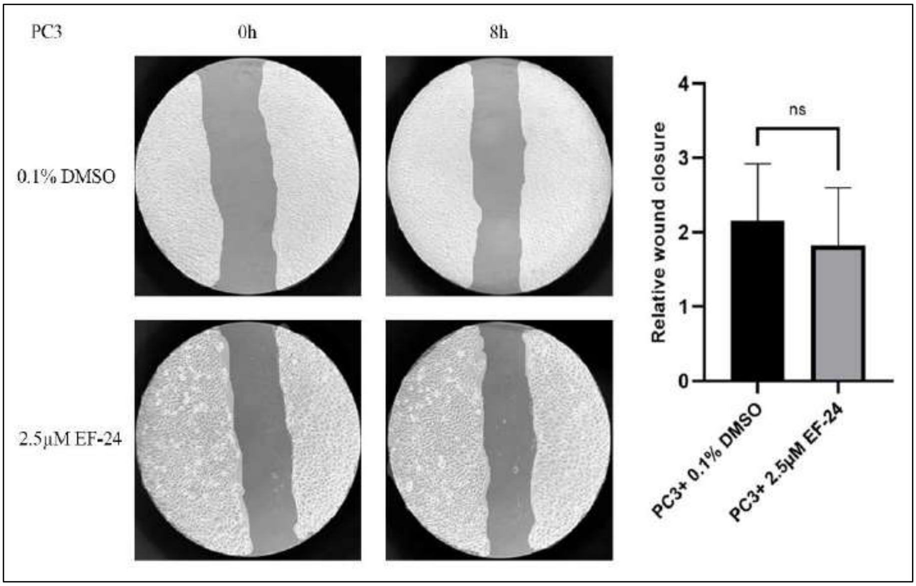

Figure 5. Measuring the effect of EF-24 on migration of PC3 cancer cell lines by wound healing assay. Right: DIC images after Scratch analysis. Left: Statistical analysis of data obtained after Scratch analysis.

The expression of the genes E-cadherin, N-cadherin, MMP-9, vimentin and twist-1 was measured in the human prostate cancer cell lines DU145 and PC3 as well as in normal prostate cancer cells PNT1A. GADPH was used as a housekeeping gene. In the qRT-PCR assay, 2.5 µM was used as the optimal concentration and 72 hours to achieve the best cytotoxic effect in cancer cells. The gene expression of E-cadherin, N-cadherin, MMP-9, vimentin and Twist-1 measured in PNT1A cells showed no change compared to 0.1% DMSO used as a control. In DU145 cells, no change in the expression of the N-cadherin, MMP-9, vimentin and Twist-1 genes was measured, while a significant increase (about 2.5-fold) in the expression of the E-cadherin gene was observed compared to the 0.1% DMSO control. In the PC3 cell line, no change in N-cadherin, vimentin and twist-1 gene expression was measured, while a significant increase (approximately 2.5-fold) in the expression of the E-cadherin and MMP-9 genes was measured compared to the 0.1% DMSO control (Figure 6).

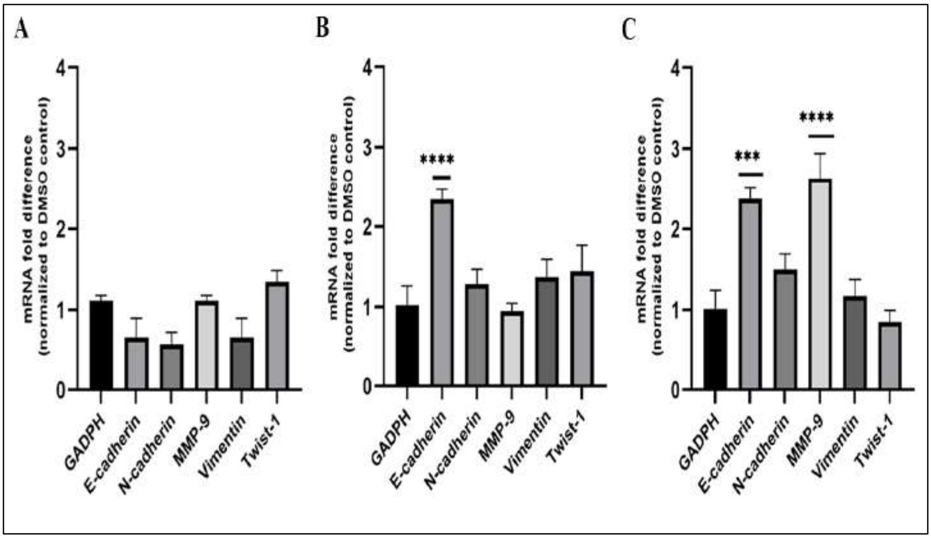

Figure 6. Measurement of genes involved in metastasis pathway by qRT-PCR after EF-24 treatment. A) PNT1A normal prostate cells, B) DU145 prostate cancer cells, C) PC3 prostate cancer cells (*p <0.05).

DISCUSSION

Prostate cancer is the most common non-cutaneous cancer in men, but metastatic prostate cancer is often incurable despite intensive and versatile therapies (Wang, et al. 2018). Therefore, the improvement of alternative therapies is becoming increasingly important. In recent years, natural ingredients and small synthetic substances derived from natural ingredients have been effective in various types of cancer, and research into some ingredients has reached the level of clinical research. One of these compounds is curcumin. It has reached phase I clinical trials for the treatment of prostate cancer. Despite its low toxicity and tolerability in cancer patients, natural ingredients such as curcumin often have low bioavailability. Therefore, small synthetic active ingredients derived from natural components with high bioavailability (60%), such as EF-24, are increasingly coming to the fore [Anand, et al. 2007; Wang, et al. 2020].

The contradictory observations of the DU-145 and PC-3 cell lines express AR mRNA prompted us to investigate whether DU-145 and PC-3 cell lines express the androgen receptor. We aim to achieve relative levels of the EF24 in DU-145 and PC-3 cell lines. PC3 is a cell line of grade IV adenocarcinomas with high metastatic potential; DU145 is a cell line of prostate carcinomas with moderate metastatic potential. In this study, PNT1A, the healthy prostate cell line with intermediate and advanced metastatic potential, was used. The reason for using these 2 kinds of cell lines is to emphasize the use of EF24 in the treatment of metastatic prostate cancer without causing a cytotoxic effect on healthy cells (Suplementary Figures 6-8, Additional background information can be found in Supplementary File 1).

In our study, the effect of the EF-24 molecule on the metastasis mechanism of prostate cancer was investigated for the first time. A two-dimensional wound healing experiment showed that EF-24 slows the migration of prostate cancer cells and thus possibly inhibits the mechanism of metastasis. At the molecular level, the expression of the E-cadherin gene increased in prostate cancer cells after treatment with EF-24. This increase indicates that the cells may become epithelial in nature. In addition to the metastasis experiments, a detailed dose investigation was also carried out in our study. The cytotoxic experiments showed that the EF-24 molecule has a dose- and time-dependent cytotoxic effect on prostate cancer cells. On the other hand, our study was the first to determine the optimal dose of EF-24 that is cytotoxic to cancer cells without having a cytotoxic effect on normal prostate cells using normal PNT1A prostate cells. These data were important for future in vivo research.

A literature review initially revealed that EF-24 induces apoptosis in cancer cells but is not cytotoxic in normal cells. Subramaniam et al. showed that EF-24 eliminated colon cancer cells but did not affect normal embryonic mouse fibroblast cells (Adams, et al. 2004). On the other hand, Chen et al. showed that EF-24, in combination with another molecule, had a cytotoxic effect on gastric cancer cells without affecting normal gastric cells (Chen, et al. 2016). In our study, the impact of EF-24 on normal prostate cells was investigated for the first time. Our results are in the same direction as the data in the literature. At effective doses in DU145 and PC3 prostate cancer cells, EF-24 PNT1A does not cause significant cytotoxicity in normal cells (Figure 1). On the other hand, docetaxel (McKeage, 2012), used in the clinic as a first-line treatment, has approximately the same cytotoxic effect on PNT1A normal prostate cells and DU145 and PC3 prostate cancer cells. These results demonstrate the potential of EF-24 to be used in combination with or as an alternative to other anti-cancer drugs.

The effect of curcumin analog EF-24 on cancer has been shown in numerous in vitro and in vivo investigations. Alp et al. showed that EF-24 inhibits the proliferation of MDA-MB-231 breast cancer cells and induces the caspase pathway (Alp, et al. 2017). Thomas et al. in lung cancer and leukemia, Yar Saglam et al. in pancreatic cancer, and Subramaniam et al. in colorectal cancer showed that EF-24 inhibits proliferation and induces apoptosis in cancer cells. Yang et al. showed that EF-24 inhibits proliferation and induces apoptosis in Saos-2 bone cancer cells, B16 melanoma cells, and DU145 prostate cancer cells (Yang, et al. 2014; Yang, et al. 2013).

Yang et al. demonstrated the effect of EF-24 on prostate cancer in vivo. It was shown that after administration of EF-24 at a dose of 200 µg/kg body weight in mice with tumors formed from DU145 cells, the size of the tumor decreased by 65% and the expression of genes involved in tumor cell proliferation decreased. In this context, the results of cytotoxicity tests performed on normal prostate cells in our study can be evaluated, and an in vivo dose calculation can be made (Yang, et al. 2014). In another study on prostate cancer with EF-24, Adams et al. showed that EF-24 was cytotoxic and activated apoptosis pathways in the DU145 cell line (Adams, et al. 2005). In this study, EF-24 was found to be effective at concentrations of 1 µM and above. On the other hand, 20 µM EF-24 was used in apoptosis assays. Our study shows that EF-24 at a dose of 20 µM has significant cytotoxicity on PNT1A normal prostate cells. Therefore, testing these studies with lower EF-24 doses will illuminate the in vivo experiments. In their study, Thomas et al. showed that EF-24 inhibited HIF-1 in PC3 prostate cancer cells at lower doses than curcumin. Inhibition of HIF-1 is related to inhibition of angiogenesis. As we have shown in our study, significant cytotoxicity is observed in PC3 cells from 24 hours at a very low dose of 1.25 µM (Thomas, et al 2008).

The effect of EF-24 on the mechanism of metastasis has been studied in various studies. Zhang et al. showed that very low doses of EF-24 inhibited migration in melanoma cells. On the other hand, EF-24 was also shown to increase the expression of E-cadherin in melanoma cells, while it decreased the expression of the mesenchymal markers N-cadherin and vimentin (Zhang, et al. 2015). In our study, a significant increase in E-cadherin expression was observed in DU145 and PC3 prostate cancer cells after treatment with 2.5 µM EF-24. This suggests that EF-24 may activate metastasis by triggering EMT in prostate cancer cells. However, no decrease in the expression of mesenchymal markers was observed in our study. This suggests that the low dose used was not sufficient in the prostate cancer cell lines DU145 and PC3, which are highly metastatic. The mechanism of action of EF-24 on hepatocellular carcinoma metastasis was investigated in the study by

Zhao et al. In this study, similar to our study, EF-24 was shown to inhibit cancer cell migration (Zhao, et al. 2016). In the most recent study, Su et al. showed that EF-24 inhibited the expression of the MMP-9 gene to prevent metastasis of nasopharyngeal carcinoma cells (Su, et al. 2023). In addition, Lee et al. showed that EF-24 inhibited the expression of MMP-9 and simultaneously inhibited the metastasis of cervical cancer cells (Lee, et al. 2023). These latter two studies differ from the results obtained in our study. In our study, an increase of MMP-9 in PC3 cells was observed after treatment with EF-24. Although there are studies in the literature stating that MMP-9 generally triggers cancer progression, it is also known that MMP-9 plays a role in inhibiting metastasis (Augoff, et al. 2022). For example, Bendrik et al. showed that MMP-9 inhibits cancer by enzymatically cleaving and activating some signaling molecules. In this study, MMP-9 was shown in vivo to inhibit breast cancer angiogenesis by cleaving collagen type XVIII and releasing the protein endostatin (Bendrik, et al. 2008).

In conclusion, EF-24 may inhibit the mechanism of metastasis in prostate cancer cells. Although the results obtained in our study show the potential of EF-24 in the treatment of prostate cancer, it needs to be supported by extensive molecular research and in vivo experiments.

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Author contributions. Conceptualization E.S., H.Y., O.O.G.; methodology E.S., H.Y., M.E.E; investigation, E.S., H.Y.; formal analysis, E.S., H.Y., M.E.E., S.S, O.O.G, writing-original draft preparation, E.S., H.Y.; writing, review, and editing, E.S., H.Y., M.E.E., S.S, O.O.G.; funding acquisition E.S. All authors have read and agreed to the published version of the manuscript.

Competing interests. Authors state no conflict of interest.

Informed consent. Not applicable.

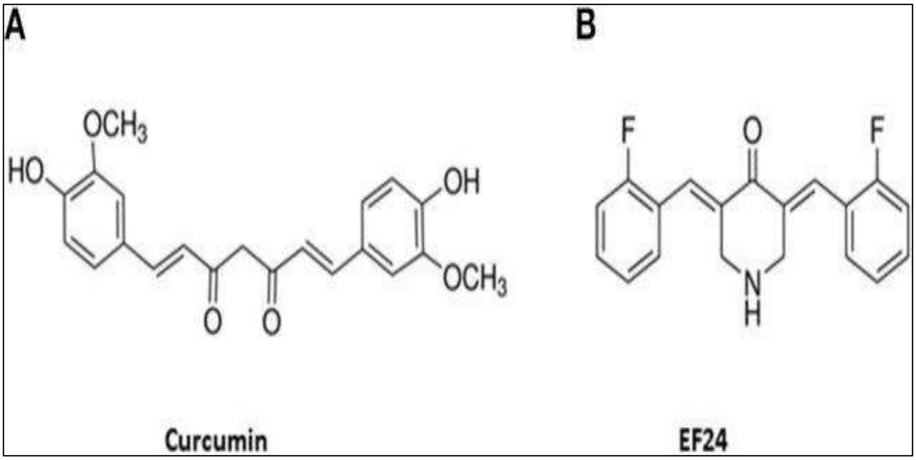
Ethical approval. Not applicable.

SUPPLEMENTARY INFORMATION

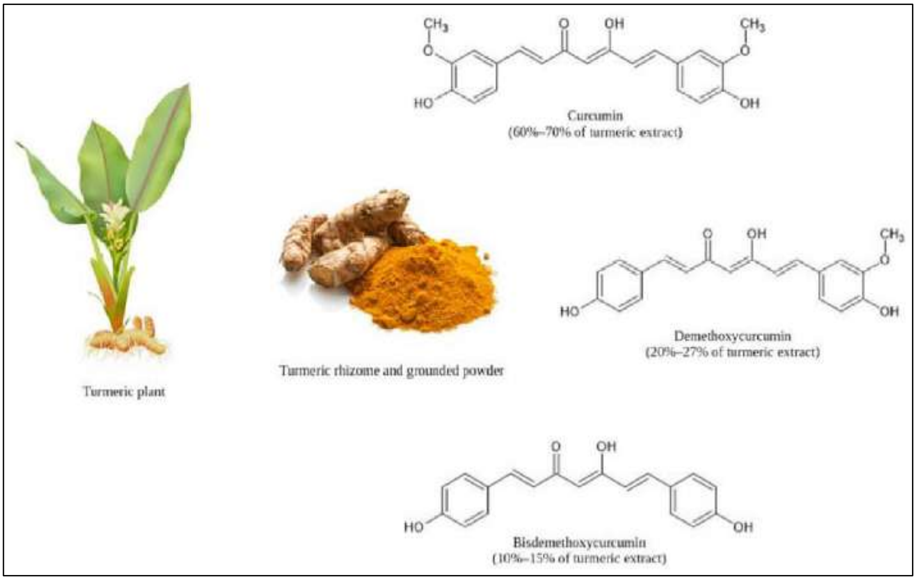
Supplementary File 1:

Curcumin is a natural polyphenolic compound, structurally classified as a β-diketone, in which two hydrogens of methane are replaced by feruloyl groups. It is the principal yellow pigment of Curcuma longa (turmeric) root and is widely recognized both as a traditional dye and a bioactive molecule. Beyond its use as a food colorant and flavoring agent, curcumin exhibits a wide spectrum of biological activities, including anti-inflammatory, antioxidant, antifungal, hepatoprotective, neuroprotective, immunomodulatory, and antineoplastic effects. It also functions as a radical scavenger, iron chelator, nutraceutical, and potential contraceptive. Mechanistically, curcumin acts as an inhibitor of several key enzymes, such as histone deacetylase (EC 3.5.1.98), aldehyde reductase (EC 1.1.1.21), shikimate dehydrogenase (EC 1.1.1.25), IMP dehydrogenase (EC 1.1.1.205), NAD(P)H dehydrogenase (quinone) (EC 1.6.5.2), thioredoxin reductase (EC 1.8.1.9), and non-specific protein-tyrosine kinases (EC 2.7.10.2). Owing to these properties, curcumin is regarded as a promising therapeutic and geroprotective agent.

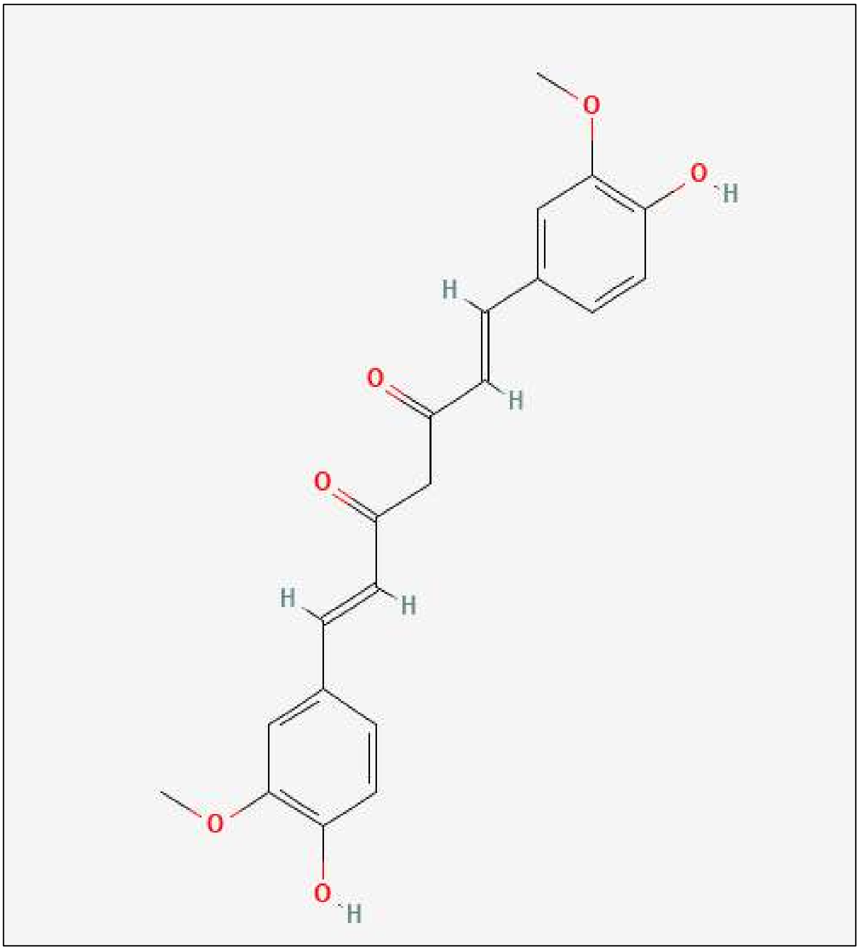
Curcumin, also called diferuloylmethane, is an active compound found in the golden spice turmeric (Curcuma longa) and in [Curcuma xanthorrhiza oil]. It is a highly pleiotropic molecule with multiple biological activities, including antibacterial, anti-inflammatory, hypoglycemic, antioxidant, wound-healing, and antimicrobial effects (PubcChem- Curcumin).



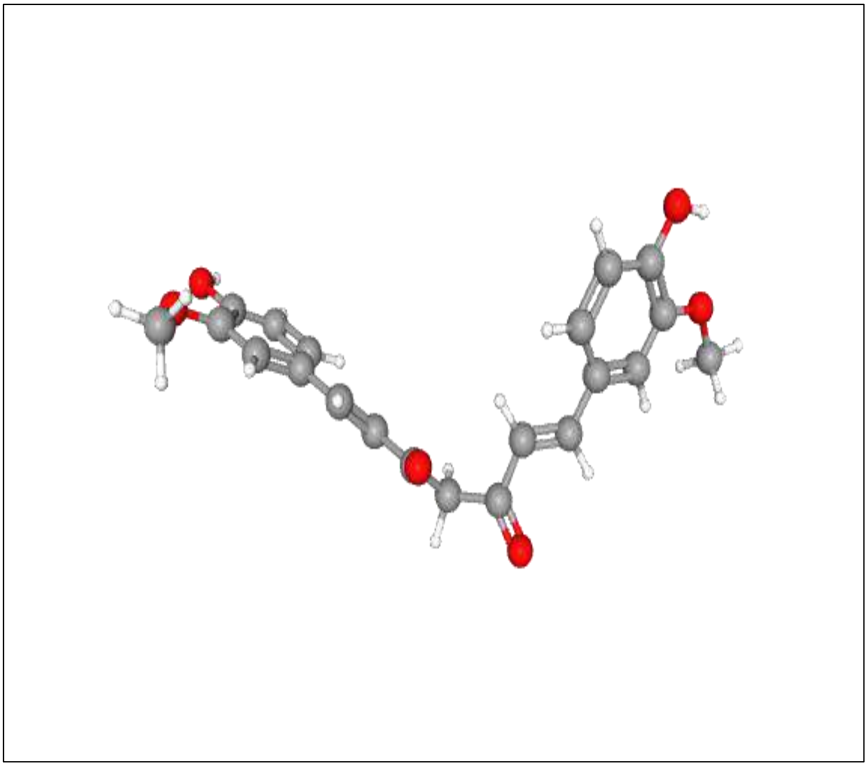
Supplementary Figure S 1. Chemical structures of curcumin (A) and EF24 (B) (He, et al. 2018).



Supplementary Figure S 2. The turmeric (Curcuma longa) plant and rhizome, and the chemical structures of the curcuminoids including curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Moetlediwa, et al. 2023).



Supplementary Figure S 3. Chemical Structure Description of Curcumin (PubChem).



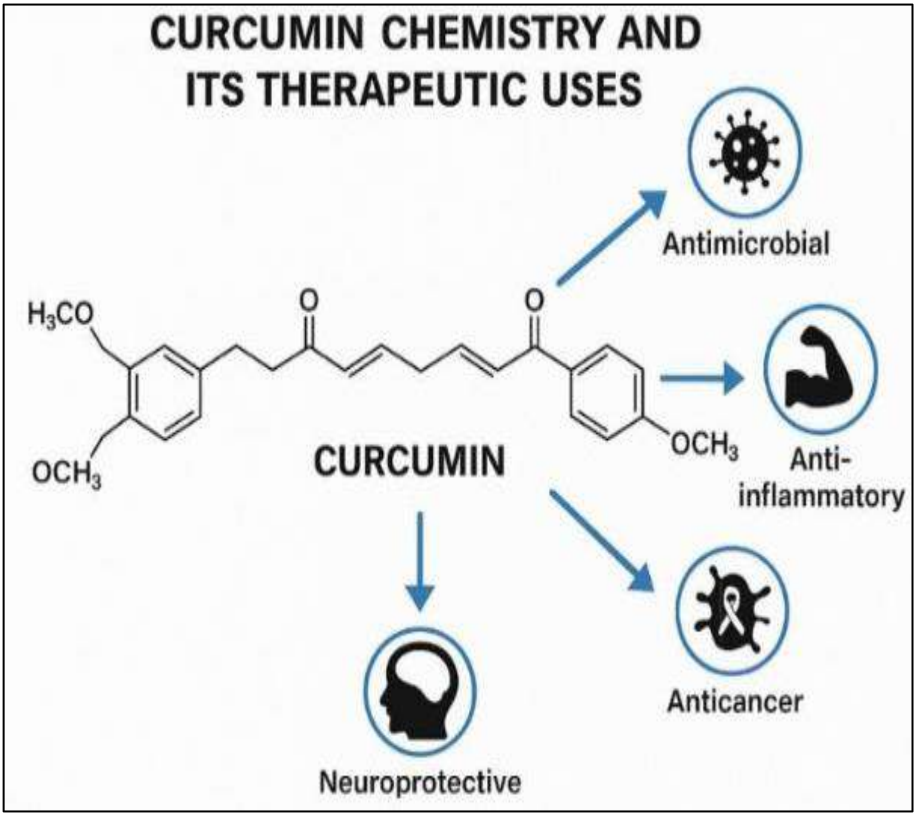
Supplementary Figure S 4. Interactive Chemical Structure Model of Curcumin (PubChem)

Biological Activities and Mechanisms of Action of EF24

Curcumin, a polyphenol derived from the rhizome of Curcuma longa and the main bioactive component of turmeric, exhibits a broad spectrum of antitumor properties across various cancer types.

Among its synthetic derivatives, EF24 has demonstrated enhanced anticancer efficacy and improved bioavailability compared to curcumin (Su et al., 2023). Curcumin has also been proposed as an alternative strategy for cancer prevention, and its potential application in targeted cancer therapy highlights its promise for more specific treatment approaches (Durgawale et al., 2025).

The main effects of EF24, which are prominent in its therapeutic applications, include anti-cancer, anti-inflammatory, and inhibition of metastasis and tumor progression. For example, one published study observed that EF24 induced apoptosis in prostate and melanoma cells by inhibiting the NF-κB signaling pathway and oncogenic miR-21, reducing tumor growth and metastasis (Yang, et al. 2013).



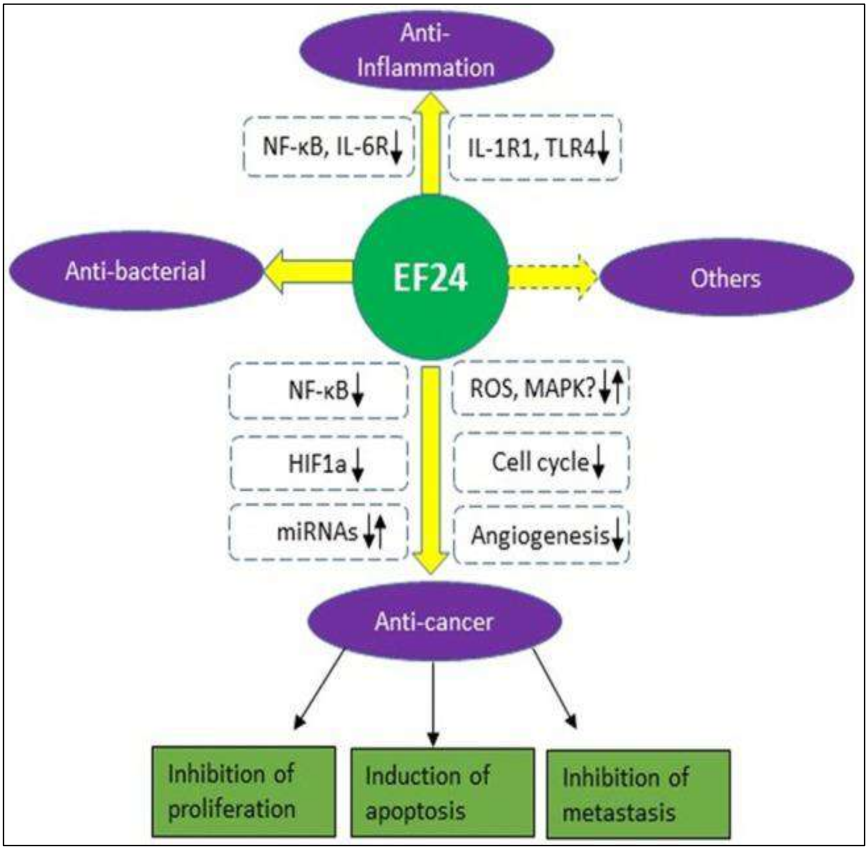
Supplementary Figure S 5. Therapeutic uses of curcumin (Pratik Durgawale, et al. 2025).

1. ***Anti-cancer Activities:*** In 2004, a series of curcumin analogs were synthesized and evaluated, among which EF24 exhibited strong cytotoxic activity against cancer cells and demonstrated greater potency than the commonly used chemotherapeutic drug cisplatin in suppressing tumor cell growth. Moreover, EF24 was identified as the most potent anti-angiogenic compound among these analogs (He, et al. 2018).

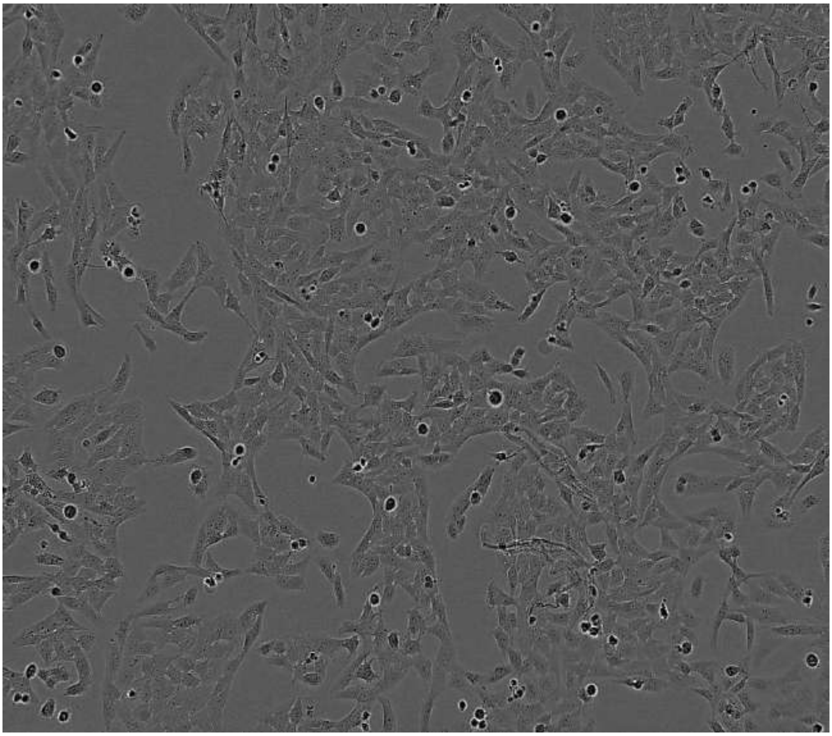
2. ***Anti-cancer Mechanisms of EF24:*** Numerous studies have indicated that EF24 suppresses cell growth by triggering cell cycle arrest and apoptosis, along with caspase-3 activation. Nevertheless, the specific signaling pathway underlying EF24's effects remained unclear until 2008, when Kasinski and colleagues first demonstrated that EF24 induces apoptosis through inhibition of the NF-κB signaling pathway (He, et al. 2018).

3. ***Regulation of HIF-1α Expression:*** Another significant function of EF24 is the regulation of HIF-1α expression, which is strongly linked to chemotherapy outcomes in cancer treatment. While curcumin suppresses HIF-1α at the transcriptional level, EF24 exerts its effect by inhibiting HIF-1α post-transcriptionally (He, et al. 2018).

4. ***Regulation of ROS Production:*** The oncogenic factor HIF-1α plays a key role in regulating aerobic glycolysis and Glut1 expression. EF24 modulates cancer cell survival by inhibiting HIF-1α activity and thereby altering glucose metabolism. Additionally, multiple studies have shown that EF24 induces reactive oxygen species (ROS) generation. Importantly, the researchers who originally designed and synthesized EF24 demonstrated its ability to promote ROS production in MDA-MB-231 human breast cancer cells and DU-145 human prostate cancer cells (He, et al. 2018).

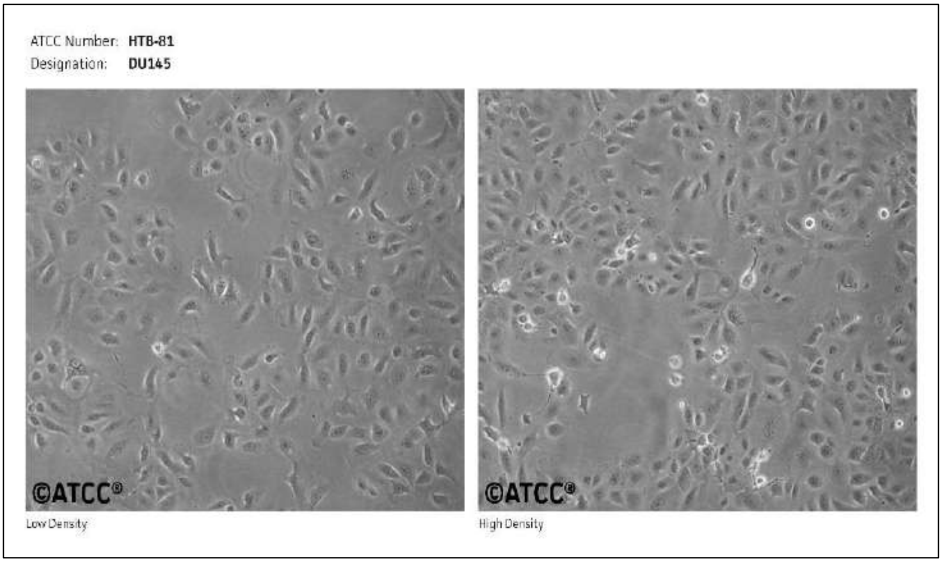


Supplementary Figure S 6. Biological activities and mechanisms of EF24 (He, et al. 2018).



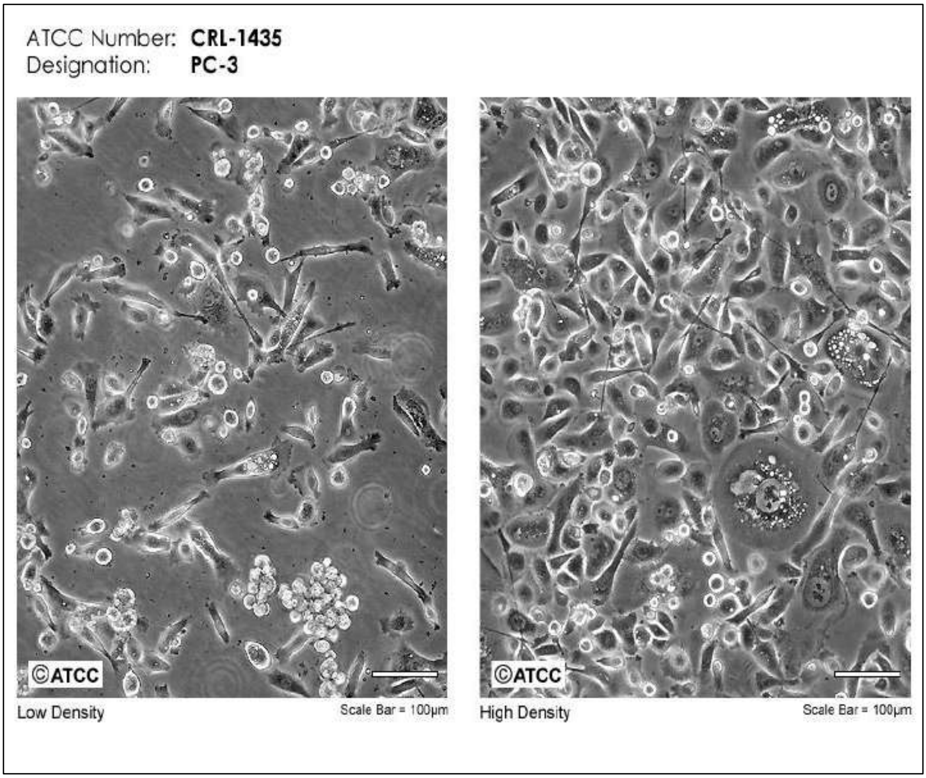
Supplementary Figure S 7. PNT1A Cell Line (UK Health Security Agency).

Normal adult prostate epithelial cells were immortalized by transfection with a plasmid containing the SV40 genome, which is defective in its replication origin. The primary culture was obtained postmortem from a 35-year-old man. The cells carried the SV40 genome and expressed large T protein. The presence of cytokeratins 8 and 18 (luminal glandular epithelial cell markers) and vimentin reflects their differentiated luminal prostate cell phenotype (UK Health Security Agency).



Supplementary Figure S 8. DU145 Cell Line (ATCC).

DU145 is a cell line with epithelial morphology that was isolated from the brain of a 69-year-old, White, male with prostate cancer (ATCC). It is a line derived from human prostate adenocarcinoma. It lacks androgen receptor (AR) expression, does not produce PSA, and has moderate metastatic potential. It is derived from brain metastases (Katsogiannou, et al. 2019).



Supplementary Figure S 9. PC3 cell line (ATCC).

PC-3 is a cell line initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old, male (ATCC). It is an aggressive, lacking AR, PSA negative and with high metastatic potential (Katsogiannou, et al. 2019).

PNT1A is classified as benign, while the other two are classified as cancerous lines, exhibiting hormone resistance and high metastatic potential. PC3 has more tumorigenic and metastatic potential than DU145. It has also been noted that the benign PNT1A line is more cluster-related to DU145 due to genetic instability (Katsogiannou, et al. 2019).

Supplementary Table S 1. Comparative Features of PNT1A, DU145, and PC3 Cell Lines (Avancès, et al 2001; Jayakumar,et al. 2014; Katsogiannou, et al. 2019).

Feature / Cell Line	PNT1A	DU145	PC3
Origin	Normal prostate epithelium immortalized with SV40	Human prostate adenocarcinoma, brain metastasis	Human prostate carcinoma, bone metastasis
Androgen Receptor (AR)	AR positive	AR negative	AR negative
Tumorigenicity	Non-tumorigenic	Moderately tumorigenic	Highly tumorigenic
Metastatic Potential	None	Moderate	High
Radioresistance/Redox	—	High GSH & Nrf2 activity → radioresistant	High ROS levels → more radiosensitive
Mechanical / Invasive Behavior	—	Moderate	High invasive capacity & contractile stress

ETHICAL STATEMENT

This study was conducted exclusively on established human prostate cancer cell lines (PC3, DU145, and PNT1A). No human participants, patient-derived materials, or animal models were involved in the experiments. Therefore, an approval from Institutional Review Board (IRB) or Animal Ethics Committee was not required. All experimental procedures were performed in compliance with institutional biosafety and biosecurity regulations, and standard laboratory practices were strictly followed.

This book has been prepared in accordance with the principles of academic integrity and ethical standards. All information presented herein has been duly cited by the author, and no violations of scientific ethics have occurred.

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