doi: 10.53446/actamednicomedia.1166837

Acta Medica Nicomedia

Cilt: 5 Sayı: 3 Ekim 2022 / Vol: 5 Issue: 3 October 2022
https://dergipark.org.tr/tr/pub/actamednicomedia

Research Article | Araştırma Makalesi

THE EFFECT OF MECLOFENAMIC ACID ON THE INVASION AND MIGRATION OF LNCaP PROSTATE CARCINOMA CELLS

MEKLOFENAMİK ASİTİN LNCaP PROSTAT KANSERİ HÜCRELERİNİN İNVAZYON VE MİGRASYONUNA ETKİSi

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Abstract

Objective: The treatment of prostate cancer has not significantly advanced despite several research to enhance early diagnosis and the introduction of innovative therapy agents. Prostate cancer metastasis is also a significant obstacle to treatment approaches. Therefore, the aim of the current investigation was to examine how the non-steroidal anti-inflammatory medicine meclofenamic acid (MA) affected the invasion and migration of LNCaP prostate cancer cells.

Methods: Firstly, the non-toxic concentrations of MA on LNCaP cells were determined by trypan blue exclusion assay. After that, the effect of MA on migration and invasion was evaluated by wound healing assay and matrigel invasion assay, respectively. Finally, western blotting was used to determine the vimentin expression level, a marker for the transition of epithelial to mesenchymal cells.

Results: The results of trypan blue exclusion assay showed that 60 and 80 µM concentrations of MA were non-toxic to the cells. The migration rate of non-treated control cells was 44.4%, while it was significantly reduced to 12.9% and 2.9% in 60 and 80 µM MA-treated groups, respectively. When the cells were treated with 80 µM MA, it significantly reduced the invasion of LNCaP cells from 52% to 30.3%. Vimentin expression was considerably decreased in MA-treated cells, according to the results of a western blot.

Conclusion: This study demonstrates for the first time that MA inhibits the migration and invasion of prostate cancer LNCaP cells by decreasing the expression level of vimentin.

Keywords: Meclofenamic acid, prostate cancer, invasion, migration, vimentin

Öz

Amaç: Prostat kanserinde erken teşhise ve yeni tedavi ajanlarının geliştirilmesine yönelik çeşitli çalışmalara rağmen, tedavide önemli bir ilerleme kaydedilememiştir. Ayrıca, prostat kanserinde metastaz tedavide büyük zorluk oluşturmaktadır. Bu nedenle, bu çalışmada nonsteroid antiinflamatuar bir ilaç olan meclofenamik asidin (MA) LNCaP prostat kanseri hücrelerinin migrasyonu ve invazyonu üzerine etkisinin araştırılması amaçlanmıştır.

Yöntem: İlk olarak, MA'nın LNCaP hücrelerine toksik olmayan konsantrasyonları, tripan mavisi analizi ile belirlendi. Daha sonra, MA'nın migrasyon ve invazyon üzerindeki etkisi, sırasıyla yara iyileştirme analizi ve matrigel invazyon analizi ile değerlendirildi. Son olarak, epitelyal-mezenkimal hücre geçişi için bir belirteç olan vimentinin ifade düzeyi western blotlama ile tespit edildi.

Bulgular: Tripan mavisi analizinin sonuçları, 60 ve 80 µM MA konsantrasyonlarının hücreler için toksik olmadığını göstermiştir. İlaç uygulanmamış kontrol hücrelerinin migrasyon oranı %44,4 iken, bu oran 60 ve 80 µM MA uygulanmış gruplarda istatistiksel olarak önemli ölçüde, sırasıyla, %12,9 ve %2,9'a düşmüştür. 80 µM MA uygulanan hücrelerde invazyon oranının %52'den %30.3'e düştüğü görülmüştür. Western blotlama sonuçları, MA verilen hücrelerde vimentin ifade seviyesinin önemli ölçüde azaldığını göstermiştir.

Sonuç: Çalışma sonucunda, MA'nın vimentin ekspresyon seviyesini azaltarak LNCaP prostat kanseri hücrelerinin migrasyonu ve invazyonunu azalttığı ilk kez gösterilmiştir.

Anahtar Kelimeler: Meklofenamik Asit, prostat kanseri, invazyon, migrasyon, vimentin

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Submitted/Başvuru: 25.08.2022 • **Accepted/Kabul:** 13.09.2022 • **Published Online/ Online Yayın:** 15.10.2022

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Introduction

Prostate cancer (PC) has become one of the most often diagnosed cancer and the second-leading cause of cancer related deaths in men globally. Age has a significant impact on both the incidence and mortality rates of PC with the average age at the time of diagnosis being sixty-six years.[1] PC develops slowly at first and needs androgens to expand. Early-stage PC responds to androgen-depletion treatment because it is androgen-sensitive. Unfortunately, following the first response to medication, the majority of patients relapse, and the condition advances from being androgen-dependent to being androgen-independent (i.e., castration-resistant PC).[2] Furthermore, castration resistance brings great challenges to the treatment of patients. Therefore, the focus of the PC battle has shifted to the creation of new therapy approaches.

Despite various studies to improve the early detection and development of novel treatment agents, no significant progress has been made in the treatment of PC. Unfortunately, the mortality rate of PC is still very high. Moreover, the metastasis of prostate carcinoma is a major challenge in clinical trials.[3] Beginning with the detachment of cancerous cells from primary tumor area, the multi-stage process of metastasis includes migration, invasion of the tissues nearby, intravasation, circulation-system-based transport, secondary-site arrest, extravasation with the help of matrix metalloproteinases (MMPs), and finally growth in the secondary organ.[4] Epithelial-to-mesenchymal cell transition (EMT) enables invasion and metastasis in cancer cells. During this transition, the expression of several proteins is regulated including extracellular matrix (ECM) proteins and enzymes involved in the breakdown of ECM.[5] Inflammation significantly contributes to the metastasis of tumors. Inflammatory proteins are accepted as potent inducers of EMT. Inflammatory cytokines including TGFβ, and TNFα activate transcription factors including NF-κB, STAT3, Smads, Twist, and Snail which promote EMT. A significant indicator of EMT is the loss or decrease of E-cadherin expression and overexpression of mesenchymal cell proteins like N-cadherin, vimentin, and MMPs.[6,7] The challenge of EMT for cancer patients is that it induces resistance to anticancer therapy.[6] Therefore, inhibition of EMT, hence migration and invasion, is important as an effective treatment strategy against cancer.

Recently, anti-inflammatory drugs have emerged as promising anticancer agents that act via several mechanisms, including inhibition of growth, cell cycle arrest, and apoptosis of several carcinoma cells.[8] Meclofenamic acid (MA), a nonsteroidal anti-inflammatory drug (NSAID), has been shown to have anti-carcinogenic effects in addition to its anti-inflammatory properties.[9-13] It has been shown that MA inhibited the growth of several cancer types including uterine cervical cancer and bladder cancer. However, it was realized that there is no study investigating the role of MA on androgen-dependent PC. Therefore, in this study, it was aimed to investigate the effect of MA on the invasion and migration of LNCaP PC cells.

Methods

Cell culture and reagents

The human PC cell line LNCaP was purchased (ATCC, Rockville, MD, USA). The cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, USA) containing high glucose, supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, and 100 µg/mL streptomycin. They were grown at 37°C in an incubator that was humidified with 5% CO2. MA was purchased (Sigma-Aldrich, M4531, USA) and prepared by dissolving 200 mg in 1 ml of DMSO. After this stock solution was obtained, different concentrations were prepared using the RPMI medium.

Cell Proliferation Assay

The proliferation of cells was determined by trypan blue exclusion (TBE) assay. The cells were seeded in triplicates at a density of 4×104 cells per well on 24-well culture plates. The cells were exposed to various concentrations of MA (20, 40, 60, 80, 100, and 120 µM) for 24, 48, and 72 hours after adhering overnight at 37°C. Harvested cells in PBS were combined with an equivalent volume of trypan blue solution following the incubation period. Live and dead cells in the mixed solution were counted by using a hemocytometer.

Wound Healing Assay

A wound healing assay was used to study the migration of LNCaP cells. 6-well plates were used to seed the cells (3x 105). Using the plate lid as a ruler, cells in each well were scraped uniformly throughout the well with a sterile 200 µl tip when they had reached 90% confluence. The cells were rinsed three times with PBS before being added to a growth medium containing 1% FBS and treated with MA at appropriate concentrations (60 and 80 µM) for 48 hours. Images were taken under a microscope immediately after wounding and 12, 24 h post wounding (Nikon, Tokyo, Japan). The wound widths were analyzed by using ImageJ.[14] Using Graphpad Prism 9.1.0, the percentage of wound closure was determined and the graph was created (La Jolla, CA, ABD). The tests were carried out three times.

Matrigel Invasion Assay

The BD BioCoat Matrigel Invasion Chambers were used to evaluate the invasive capacity of LNCaP cells (Corning, BD No. 354480, USA). 500 µl of serum-free media was used to hydrate the 8 mm porous membranes coated with matrigel for 2 hours. After the hydration period, 1x105 cells per insert in 500 µl of RPMI were put into upper chambers, and 750 µl of RPMI with 20% FBS into the lower chambers. Non-invasive cells on top of the membrane were eliminated using cotton swabs following a 24 h incubation period at 37°C. Using a diff-quick staining kit (Polysciences, 26419-8), the cells that invaded

the other side of the membranes were stained after fixation. After cells were fixed with solution A for 4 minutes, they were stained with solution B and C, respectively, for 4 minutes. Finally, the cells were gently rinsed twice with distilled water. Under a microscope, the stained cells were counted and photographed in five randomly chosen fields. The same procedure was followed by using control inserts that were not coated with matrigel. All the experiments were performed in triplicate. The percentage of invasion was calculated using the formula as suggested by the manufacturer (Corning, BD No. 354480, USA)

Western Blotting

Total proteins were extracted as previously described.[15] 20 µg of protein from each sample was separated on 12% SDS-PAGE and then transferred to PVDF membranes (Roche, Indianapolis, IN, USA). The membrane was blocked by non-fat milk and incubated with goat anti-mouse primary antibodies against vimentin (1:2000, Clone E-5, sc-373717, Santa Cruz, USA) overnight at 4°C. The protein bands were identified by ECL after one hour of incubation with HRP-conjugated goat anti-mouse secondary antibody (1:1000 dilution). For band normalization, the same membrane was stripped and treated with beta-actin (1:2000, ACTBD, sc-81178, Santa Cruz, USA). ImageJ was used for band analysis.[14]

Statistical Analysis

The data were statistically analyzed using GraphPad Prism 9.1.0 (La Jolla, CA, ABD) and reported as the mean ± standard deviation of three independent experiments. For multiple comparisons, one-way analysis of variance (ANOVA) and Tukey's test were utilized. In order to compare groups, a two-way ANOVA analysis was also carried out. p values under 0.05 were considered statistically significant.

Results

MA reduces prostate cancer cell proliferation

LNCaP cells were exposed to increasing concentrations of MA (20, 40, 60, 80, 100, and 120 µM) for different time periods (24, 48, and 72 hours) to compare their relative sensitivities to the growth inhibition of MA. It was aimed to determine the suitable concentrations and exposure time for further experiments. The outcomes showed that MA inhibited the proliferation of LNCaP cells in a dose- and time-dependent manner. The viability of cells was statistically significantly reduced ($p<0.05$) at 40 µM and higher concentrations of MA after 24 h incubation, whereas significantly reduced viability ($p<0.05$) was detected at 20 µM and higher concentrations for 72 h treatment (Figure 1). 80 µM of MA treatment for 24 h decreased the viability of LNCaP cells to 49.2%, while 60 µM reduced the viability to 54.1% when treated for 48 h. Thus, 60 and 80 µM concentrations of MA were chosen for further analysis to evaluate the effect of the drug on the migration of LNCaP cells.

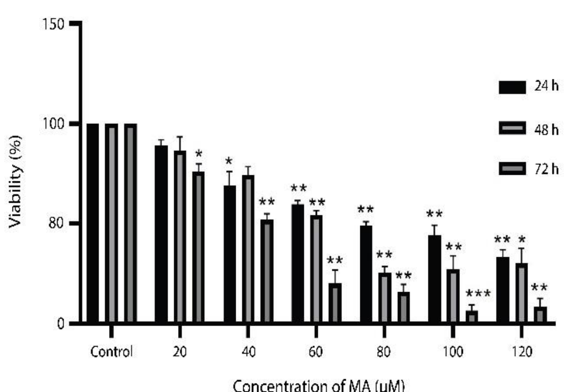

Figure 1. The effects of MA on the proliferation of LNCaP cells. Relative cell viability was measured after treatment with 20, 40, 60, 80, 100 and 120 µM of MA (*$p<0.05$, ** $p<0.005$, ***$p<0.0005$).

MA inhibits the migration and invasion of LNCaP cells

To analyze the suppressive effect of MA on the migration of LNCaP cells, a wound-healing assay was performed. As shown in Figure 2a, treatment with MA suppressed the migration of these cells. Furthermore, this enormous inhibitory impact was dose- and time-dependent. The migration rate of non-treated control cells was 44.4%, while it was 12.9% and 2.9% in 60 and 80 µM MA treated groups, respectively. The initial wound distances were 0.84, 0.70, and 0.70 mm for control, 60, and 80 µM MA treated groups, respectively. The distance was statistically significantly reduced ($p<0.005$) to 0.47 mm in the control group and to 0.61 ($p<0.05$) in the 60 µM MA treated group (Figure 2b). However, there was not statistically significant drop in wound distance in the 80 µM MA treated group. Thus, 80 µM MA treatment for 24 hours inhibited the migration of LNCaP cells.

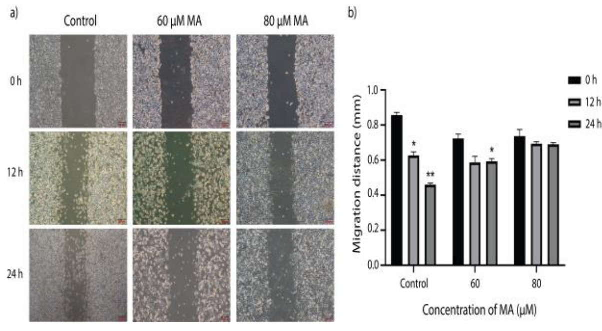

Figure 2. The effect of MA on LNCaP cell migration. The migration of LNCaP cells was evaluated using wound healing assay. a) After 12 and 24 h of incubation, the scratches were photographed (magnification, ×40) and b) the migration distances were measured (*$p<0.05$, ** $p<0.005$). The data are presented as mean ± standard deviation of three independent experiments.

We further examined the effect of MA on the invasion of LNCaP cells using the Matrigel invasion assay. Since treatment by 80 µM MA for 24 h inhibited the migration

of cells, only this concentration and incubation time were used for the invasion assay. The number of invasive cells was determined and it was found that the invasion of LNCaP cells into collagen-coated basement membrane was inhibited by the presence of 80 µM MA (Figure 3a). MA statistically significantly ($p<0.005$) reduced the invasion of LNCaP cells from 52% to 30.3% (Figure 3b). Thus, the invasion capacity was inhibited by 21% upon drug treatment. These observations indicate that critical doses of MA may provide considerable inhibition of migration and invasion of LNCaP cells.

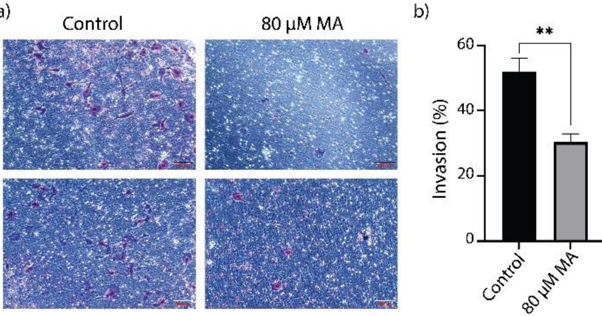

Figure 3. The effect of MA on LNCaP cell invasion. The invasion of LNCaP cells was assessed using Transwell invasion assay. a) After 80 µM MA treatment for 24 h, the cells invading the matrigel membranes were photographed and b) invaded cells were calculated (**$p<0.005$). All experiments were repeated three times. Data were expressed as means ± S.E.M.

MA decreases vimentin expression in LNCaP cells

EMT provide cancerous cells to undergo invasion and metastasis. An important hallmark of EMT is the upregulation of mesenchymal cell proteins including MMPs and vimentin.[6] To verify the inhibitory effect of MA on migration and invasion, a change in vimentin level, an EMT marker, was detected by Western blotting (Figure 4a).

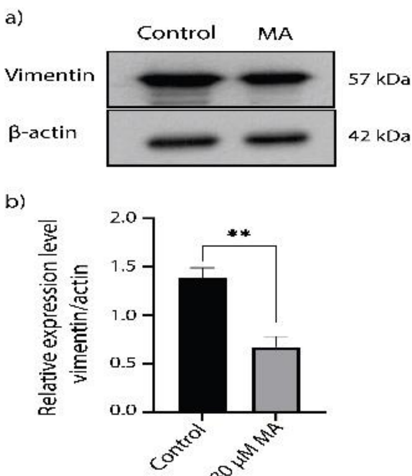

Figure 4. a) Representative Western Blot images and b) the relative expression level of vimentin in 80 µM MA treated and non-treated cells. 20 µg proteins per lane were loaded from each sample. The band intensities were measured by performing densitometric analysis using ImageJ software.

The findings of the experiment showed that the expression level of vimentin had significantly decreased ($p<0.005$) in MA-treated cells when compared to the non-treated control cells (Figure 4b). Treatment with 80 µM MA for 24 hours caused a 2.1-fold decrease in vimentin protein level. The data implies that reduction of LNCaP cells' invasion and migration following MA treatment may be caused by down-regulation of vimentin.

Discussion

After PC relapses, it progresses to an androgen-independent form, which is more resistant to therapy.[2] Various biological agents have not become effective for the treatment.[8,16,17] Thus, the development of novel treatment regimens for PC is needed.

When working on PC, it is important to take into account the many characteristics of PC cells, such as androgen dependence. An active androgen receptor causes the LNCaP cell line to release prostate-specific antigen and express special membrane antigen and prostatic acid phosphatase. This cell line, which developed from a PC patient's lymph node metastasis, is tumorigenic and metastatic in castrated hosts.[18] It is also proposed that LNCaP cells are a better model for simulating cancer development *in vivo.*[19] Both for these reasons and since the effects of MA on the migration and invasion of these cells have never been studied, it was decided to use LNCaP cells in this study.

Meclofenamic acid (MA), an NSAID, has been shown to have anti-carcinogenic effects on various types of cancer.[9-11,20] However, the effect of the drug on LNCaP cell migration and invasion has not been fully elucidated. In the present study, its effect on migration and invasion was assessed by wound healing assay and matrigel invasion assay. Before starting these assays, the effect of MA on cell proliferation was investigated. TBE assay results showed that MA exerted an anti-proliferative effect on LNCaP cells in a dose- and time-dependent manner. In our previous study, the effect of MA on healthy/ normal PC cell line PNT1A was assessed and it was found to be non-toxic to these normal cells.[21] Overall, these findings suggest that MA could be safely used as an anti-carcinogenic agent in studies conducted on LNCaP cells.

The metastasis of prostate carcinoma is a major challenge in clinical trials.[3] Two major steps of metastasis, migration, and invasion, have been tried to be inhibited using several agents in PC by targeting multiple pathways.[2,22,23] A new histone deacetylase inhibitor MHY219[3], 5-alpha reductase inhibitor finasteride[24], core3 O-Glycan Synthase[25], Flavonoids from Orostachys japonicus A. Berger[26], an EP4 Antagonist ONO-AE3-208[2], green tea polyphenol EGCG[17] and cordycepin (3'-deoxyadenosine) and a major bioactive compound of Cordyceps militaris[4] were reported to inhibit the migration and invasion of the LNCaP cells.

There are just a few researches on the anti-carcinogenic effects of MA on PC.[9,12,27] In their *in vitro* and *in vivo* study, Soriano-Hernandez et al. showed that this drug increases survival by reducing tumor growth.[27] The analysis of histological changes in the MA-treated androgen-independent PC3 cells revealed that MA had a slowing effect on cell growth.[9] In the PC-3 and LNCaP cells, MA has been reported to increase the effect of simvastatin alone in reducing cell proliferation and migration. Furthermore, MA was found to inhibit IGF1-induced Akt activation and has been suggested to be particularly effective in the treatment of castration-resistant PC.[12] Recently, Guzman-Esquivel et al. conducted a prospective and randomized clinical trial.[28] They reported that MA treatment slowed the course of the castration-resistant PC, enhanced the life quality and raised body mass index in individuals with castration-resistant PC. The outcomes of our research are consistent with the aforementioned findings. Additionally, for the first time, it was shown that MA could inhibit the migration and invasion of PC LNCaP cells. Treatment of cells with 80 µM MA for 24 hours had statistically significantly inhibited the migration in comparison to non-treated cells. Although the lower dose of MA (60 µM) significantly inhibited migration, it did not inhibit as much as in the 80 µM-treated group, which seems to be a result of an adaptive response of the cells to the low-dose MA environment. Sekine et al. evaluated the combination impact of simvastatin and MA in the PC3 cells, but not in LNCaP cells. They found that administration with simvastatin or MA alone decreased cell growth and migration and reported that the effect of the drug is increased when used in combination.[12] Thus, further studies including a combinatory agent might improve the effect of MA on LNCaP cells. To investigate the effect of MA on the invasion of LNCaP cells, the Matrigel invasion assay was used. The assay allows determining the number of invasive cells which penetrate through a matrigel membrane. The results of the assay showed that MA statistically significantly reduced the invasion of LNCaP cells from 52% to 30.3%. This is the first finding showing the inhibitory effect of MA on PC. Taken together with the results of migration, it could be postulated that MA inhibits the migration and invasion of LNCaP cells.

A key protein related to invasion and migration is vimentin. This protein is a type III mesenchymal filament-forming structural cytoskeletal protein in mammals. Vimentin is overexpressed during epithelial-to-mesenchymal cell transition and abnormal expression is related well with upregulated cell migration or invasion both in the fetus and in malignancy.[6,29] Various investigations reported that vimentin might have an effect on the invasion and migration of PC cells and is a potential marker for predicting metastatic and aggressive PC.[30,31] In our research, to further validate the inhibitory effect of MA on migration and invasion, the expression level of vimentin was investigated. The outcome of Western blotting showed that the level of vimentin was statistically significantly reduced. According to their characteristics of metastatic growth, LNCaP cells typically exhibit increased vimentin expression. Thus, the decrease in the expression level of vimentin verified the potential of MA as an inhibitor of migration and invasion in LNCaP cells.

Conclusion

In conclusion, we report for the first time that MA inhibits the invasion and migration of prostate carcinoma LNCaP cells by regulating the expression level of vimentin. When the cells were treated with 80 µM of MA for 24 hours, the level of the protein was statistically significantly decreased, which demonstrate the inhibitory effect of MA. The data indicate that MA might be used as a potential therapeutic drug in the treatment of metastatic cancers. Elucidating the underlying mechanisms and signaling pathways will open up avenues for developing novel therapeutic strategies.

Compliance with Ethical Standards

Ethics approval was not required for this study.

Conflict of Interest

None declared.

Author Contribution

AK and SY: Study design, performed the experiments; AK: Data interpretation; SY: Literature scanning; AK and SY: Writing manuscript; AK: Critical revision.

Financial Disclosure

No financial support was received for this research.

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