MCBU SBED
MANİSA CELAL BAYAR ÜNİVERSİTESİ SAĞLIK BİLİMLERİ ENSTİTÜSÜ DERGİSİ
MANISA CELAL BAYAR UNIVERSITY JOURNAL OF INSTITUTE OF HEALTH SCIENCE
ISSN: 2147-9607

ARAŞTIRMA MAKALESİ
RESEARCH ARTICLE
CBU-SBED, 2023, 10 (2): 90-97.

Kafein Sisplatinin Prostat Kanseri Hücreleri Üzerindeki Anti-Tümörijenik Etkisini Güçlendirebilir

Caffeine May Potentiate the Anti-tumorigenic Effect of Cisplatin on Prostate Cancer Cells

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Gönderim Tarihi / Received:30.10.2022
Kabul Tarihi / Accepted: 05.04.2023
DOI: 10.34087/cbusbed.1196678

Öz

Giriş ve Amaç: Günümüzde prostat kanseri (PCa) tedavisinde cerrahi, androjen ablasyon tedavisi ve kemoterapi gibi çok sayıda yaklaşım bulunmaktadır. Ancak, androjen reseptörü (AR) hedefli terapiler veya mevcut terapilerin doğal bileşiklerle kombinasyonunun geliştirilmesine halen ihtiyaç duyulmaktadır. Sisplatin çeşitli solid tümörlerin tedavisinde yaygın olarak kullanılan ilk platin bazlı kemoterapötik ajanlardan birisidir. Kafein (Cfn) altmıştan fazla bitki türünde doğal olarak bulunan ve dünya çapında en sık tüketilen nöroaktif doğal ürün olan ksantin türevi bir alkaloittir. Anti-oksidan, anti-inflamatuar ve anti-kanser gibi çeşitli biyokimyasal etkilere sahiptir. Bu çalışmada, yaygın olarak kullanılan bir kemoterapötik ajan olan sisplatinin kafein ile kombinasyonunun PCa hücreleri üzerindeki etkilerini araştırdık.

Gereç ve Yöntemler: Total-p38 MAPK, fosforile-(Thr180/Tyr182) p38 MAPK, total NF-κB, fosforile-(Ser536) NF-κB, Siklin A2, Siklin B1, Siklin E1, AR, PSA ve VEGF-A protein ifadesi düzeyleri immünoblotlama çalışmaları ile insan prostat kanseri hücre hattı olan LNCaP hücrelerinde incelenmiştir.

Sonuçlar: Elde ettiğimiz sonuçlar, Cfn'nin siklin A2, B1 ve E1 düzeylerini azaltarak, androjenik sinyal ile ilişkili AR ve PSA düzeylerini ve anjiyojenik düzenleyici VEGF-A protein düzeylerini baskılayarak sisplatinin LNCaP hücreleri üzerindeki etkisini sinerjistik olarak doz-bağımlı bir şekilde arttırdığını gösterdi. Ayrıca, sisplatin kaynaklı p38 MAPK ve NF-κB aktivasyonunun Cfn uygulaması tarafından baskılandığını belirlendi.

Tartışma: Sonuçlarımız, sisplatin ve Cfn'nin kombine kullanımının kemoterapötiklerin toksik dozunu azaltarak PCa tedavisinde etkili bir terapötik yaklaşım sunabileceğini göstermektedir.

Anahtar Kelimeler: Androjen reseptörü, Kafein, Prostat kanseri, Sisplatin.

Abstract

Objective: Today, there are numerous approaches for prostate cancer (PCa) treatment, such as surgery, androgen ablation therapy and chemotherapy. However, there is still a need to develop new androgen receptor (AR)-targeted therapies or a combination of existing therapies with natural compounds. Cisplatin is the first platinum-based anti-cancer drug which is one of the widely used chemotherapeutic agents in the treatment of various solid tumors. Caffeine (Cfn) is a xanthine-derived alkaloid that occurs naturally in more than sixty plant species and is the most frequently consumed neuroactive natural product globally. It has various biochemical effects, including anti-oxidant, anti-inflammatory and anti-cancer. In the present study, we investigated the effects of the widely used chemotherapeutic agent cisplatin and its combination with caffeine on PCa cells.

Materials and Methods: We examined the protein expression level of total-p38 mitogen-activated protein kinase (MAPK) phospho-(Thr180/Tyr182) p38 MAPK, total nuclear factor-κB (NF-κB), phospho-(Ser536) NF-κB, cyclin A2, cyclin B1, cyclin E1, androgen receptor (AR), prostate-specific antigen (PSA) and vascular endothelial growth factor A (VEGF-A) in human prostate cancer cell line LNCaP by immunoblotting assay.
Results: Our results indicated that Cfn synergistically increased the effect of cisplatin on LNCaP cells in a dose-dependent manner by decreasing the cyclin A2, B1 and E1 levels, reducing androgenic signal-related AR and PSA levels and angiogenic regulator VEGF-A levels. Also, we found that cisplatin-induced p38 MAPK and NF-κB activation were suppressed by Cfn administration.
Conclusion: Our results suggest that combinatory usage of cisplatin and the cost-effective agent Cfn may exhibit an effective therapeutic approach in the treatment of PCa by reducing the toxic dose of chemotherapeutics.

Keywords: Androgen receptor, Caffeine, Cisplatin, Prostate cancer.

1. Introductions

Prostate cancer (PCa) is the second most common cancer type, which comprises 14.1% of cancers in men and age, race, genetics, family history, obesity and smoking are the most common risk factors for the development of a prostate tumor. Today, there are numerous approaches for prostate cancer treatment, such as surgery, androgen ablation therapy, chemotherapy and radiotherapy [1-8]. Androgen ablation therapy which aims to reduce the levels of serum androgens and inhibit androgen receptor (AR), is the first-line treatment in PCa. However, PCa cells may transform into a castration-resistant form following treatment thereby treatment may fail [9-11]. Therefore, developing new AR-targeted therapies or combining existing treatments with natural compounds is crucial for preventing resistance.

Cisplatin [cis-diamminedichloroplatinum(II)] is the first platinum-based anti-cancer drug, one of the widely used chemotherapeutic agents in the treatment of various solid tumors, including ovarian, bladder, lung and testicular [12,13]. However, acquired resistance against chemotherapeutics, including cisplatin, constitutes the main hindrance to effective treatment [14]. Besides that, identifying the molecular mechanisms underlying acquired resistance or researching the combinational approaches that target preventing resistance is essential for improving the efficacy of current chemotherapeutics [15].

Caffeine (1,3,7 trimethylxanthine) (Cfn) is a xanthine-derived alkaloid that occurs naturally in over sixty plant species, such as cocoa beans, cola seeds, coffee, and tea leaves and contains various bioactive compounds. Moreover, it has well-known anti-oxidant, pro-oxidant properties and anti-cancer effects [16-20]. It has been shown that Cfn promoted cell death in cancer cells and prevented mutagenesis [18]. Due to Cfn being an easily accessible natural product exhibiting broad biochemical effects, may be an essential source of biochemical compounds that may contribute to protection against PCa [21]. The exact molecular mechanism of Cfn on PCa has not been broadly investigated yet. Herein, we combined cisplatin and Cfn to examine potential anti-cancer effects in PCa.

Mitogen-activated protein kinases (MAPKs) are one of the crucial components of the intracellular signaling system that controls various physiological processes, such as cell proliferation, cell death, differentiation, and migration [22]. There are four main cascades that share MAPK signaling pathways, including the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino-terminal kinases (JNK1/2/3), p38-MAPK and ERK5. Although all kinases work in cascades, detailed molecular interaction patterns have not been known yet, because they interact with various cellular signals [23]. Studies demonstrated that expression profiles of MAPKs changed depending on the cancer type. Also, the link between the activation of MAPKs and cisplatin resistance has been reported in numerous studies [24]. Moreover, protein kinase signaling pathways have been demonstrated to affect AR signaling. It is thought that MAPK signaling pathway activation may affect the AR-mediated gene regulation in PCa cells [25,26].

The nuclear factor kappa B (NF-κB) transcription factor regulates innate and adaptive immune responses. Due to the understanding of its supportive role in cancer progression, the NF-κB signaling pathway is one of the recently popularized signaling mechanisms in cancer development and regulation. It controls the angiogenesis and invasion in tumor cells; therefore, it is among the main targets of new-era chemotherapeutics [27]. In addition, MAPK and NF-κB signaling pathways are interrelated in inflammation-based cytokine release [28]. Herein, we investigated the effects of Cfn alone or combined with cisplatin on AR signaling which is crucial for prostate carcinogenesis by acting through the MAPK and NF-κB signaling pathways.

In this study, we investigated the effects of the widely used chemotherapeutic agent cisplatin and its combination with Cfn on PCa cells. While cisplatin led to activation of MAPK/NF-κB signaling and decreased Vascular endothelial growth factor A (VEGF-A) levels, co-treatment with Cfn and cisplatin suppressed MAPK/NF-κB activation, decreased cell cycle proteins, VEGF-A, AR and prostate-specific antigen (PSA) levels. Collectively, our results suggest that combinatory usage of cisplatin and the cost-effective agent Cfn may be an

effective therapeutic approach in the treatment of PCa.

2. Materials and Methods

2.1 Materials: Cell culture plastic materials were obtained from Sarsdeth. Fetal bovine serum (FBS), L-Glutamine and other cell culture grade growth requirements were obtained from Capricorn Scientific. Roswell Park Memorial Institute (RPMI) 1640 Medium was purchased from LONZA Bioscience. Caffeine (#C0750) and cisplatin (#232120) were purchased from Sigma Aldrich. Rabbit polyclonal antibodies p38 MAPK (#9212)(1:1000), phospho-p38 (Thr180/Ty3182) MAPK (#9211)(1:1000), NF-κB (#8242)(1:2000), cyclin A2 (#4656)(1:1000), cyclin B1 (#12231)(1:1000) and cyclin E1 (#4129)(1:1000) were obtained from Cell-signaling Technologies. Rabbit polyclonal antibodies AR (#22089-1-AP)(1:3000) and PSA (#10679-1-AP)(1:2500) were provided from Proteintech. Rabbit polyclonal VEGF-A (#E-AB-40004)(1:1000) was purchased from Elabscience. Phospho NF-κB p65 (Ser536) (#BT-PHS00191)(1:1000) was obtained from Bioassay Technology Laboratory. Monoclonal mouse beta-actin antibody (#A5316)(1:10000) was purchased from Sigma Aldrich. HRP-conjugated goat anti-mouse (#31430)(1:5000) or goat anti-rabbit (#31460)(1:5000) IgG (H+L) was provided from Thermo Scientific. Caffeine and cisplatin were dissolved in sterile protease and nuclease-free ultrapure H_2O (Gibco®).

2.2 Cell Culture: Human epithelial prostate adenocarcinoma cell line LNCaP (CRL-1740™) was obtained from American Type Tissue Culture (ATCC). The cells were propagated in RPMI 1640 Medium completed with 10% FBS and 2 mM L-glutamine under conventional cell culture conditions (5% CO_2 and 95% air at a constant temperature of 37 °C). The absence of mycoplasma contamination was regularly proved by MycoAlert™ Mycoplasma Detection Kit (Lonza).

2.3 WST-1 assay: LNCaP cells were seeded in a 96-well plate with 10,000 cells per well. After 24 hours, agents were applied to the cells for 48 hours. Cell viability was examined with the Cell Counting Kit-8 (CCK-8) assay following the manufacturer's instructions (Abbkine #KTA1020). The absorbances were measured at 450 nm using a microplate reader (Biotek, Epoch2). The results were presented as % cell viability in the graph.

2.4 Western blotting: Protein expression was performed as described before [29]. Cell lysates were prepared with Radioimmunoprecipitation assay (RIPA) buffer and then insoluble materials were removed by centrifugation at 14.000 rpm for 30 min at 4°C. The concentration of isolated protein content was determined by bicinchoninic acid assay (BCA) kit (Thermo Scientific). Typically, 25-40 µg of total protein was used in the protein analysis. Samples were mixed with 4x Laemmli buffer (Bio-Rad) and denatured at 95°C for 5 min. Protein samples were separated on hand-cast polyacrylamide gels and then transferred to an Immun-blot® PVDF membrane (Bio-Rad). Target proteins were marked using target specific-primary antibodies and visualized by Pierce™ ECL Substrate (Thermo Scientific) using ChemiDoc XRS+ system (Bio-Rad) following the classical immunoblotting steps. Densitometric analysis of protein bands was performed with ImageJ software.

2.5 Statistical Analysis: The results were presented as mean ± standard deviation (SD). The statistical significance of the differences between the groups was determined by the Student t-test with a minimum confidence interval of 95% using GraphPad Prism 7 software. The value of $p<0.05$ was considered significant.

3. Results and Discussion

3.1 Result

3.1.1 The effect of co-administration of Caffeine and cisplatin on LNCaP cell viability

To evaluate the anti-cancer effect of Cfn on cisplatin, we first examined its effect on cell viability by WST-1 based cell viability assay. Cfn and cisplatin doses used in the study were determined by evaluating the literature data [30-34]. Our results indicated that 10µM cisplatin administration significantly suppressed the viability of LNCaP cells compared to the control group. Combined application of cisplatin with Cfn doses (5, 7.5 and 15mM) significantly improved the reducing effect of cisplatin on cell viability in a dose-dependent manner compared to the cisplatin-treated group alone (Figure 1). These results suggested that Cfn enhanced the anticancer activity of cisplatin in LNCaP cells.

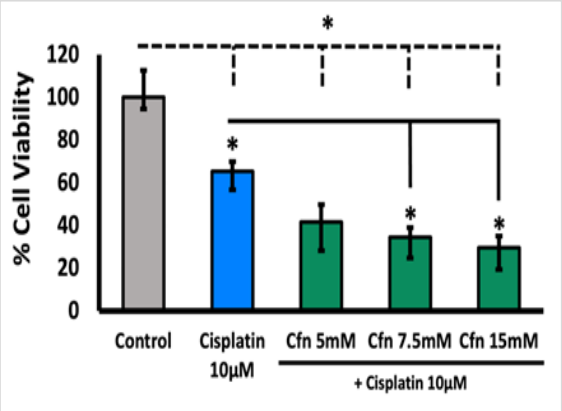

Figure 1.

Investigation of the effect of Caffeine on the anti-cancer activity of cisplatin in LNCaP cells. Cells were treated with vehicle, 10µM cisplatin or its combination with 5mM, 7.5mM and 15mM Cfn for 48 h and then the cell viability was measured using

WST-based cell viability assay. % cell viability was graphed in terms of fold increase. (n=3) (* p<0.05)

3.1.2 Caffeine treatment reduces cisplatin-induced MAPK activation.

We evaluated the effect of cisplatin and its combination with Cfn on MAPK activation in LNCaP cells by immunoblotting assay. Our results indicated that cisplatin and Cfn did not affect the total p38 MAPK levels, whereas phosphorylated at Thr180/Tyr182 p38 MAPK levels increased through cisplatin treatment compared to the control group. Combined treatment with cisplatin and Cfn remarkably decreased the phosphorylated p38 MAPK levels in a dose-dependent manner compared to the cisplatin-treated group alone (Figure 2).

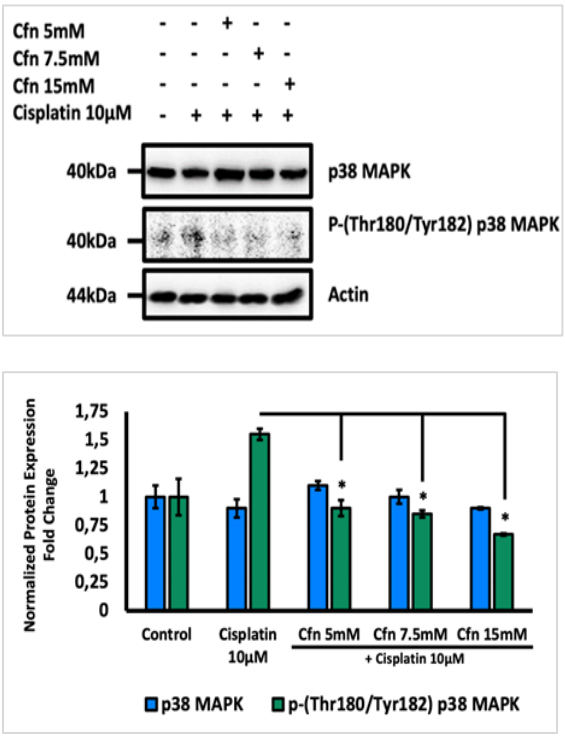

Figure 2.

Evaluation of the effect of caffeine on p38 MAPK activation. Cells were treated with vehicle, 10µM cisplatin or its combination with 5mM, 7.5mM and 15mM Cfn for 24 h and then total p38 MAPK, phosphor (Thr180/Tyr182) p38 MAPK protein levels were examined by immunoblotting. Beta-actin was used as a loading control. Changes in protein expressions were graphed in terms of fold increase. (n=3) (* p<0.05)

3.1.3 Caffeine blocks the cisplatin-induced NF-κB signaling.

To evaluate the effect of the cisplatin and combining with Cfn on NF-κB signaling, we examined the total and phosphorylated at Ser536 position NF-κB levels by immunoblotting studies. Our results indicated that cisplatin treatment strongly increased the phosphorylated NF-κB levels in LNCaP cells. Combined application of cisplatin with Cfn efficiently reversed the Cfn-induced phosphorylated NF-κB levels in a dose-dependent manner compared with the cisplatin-treated group (Figure 3).

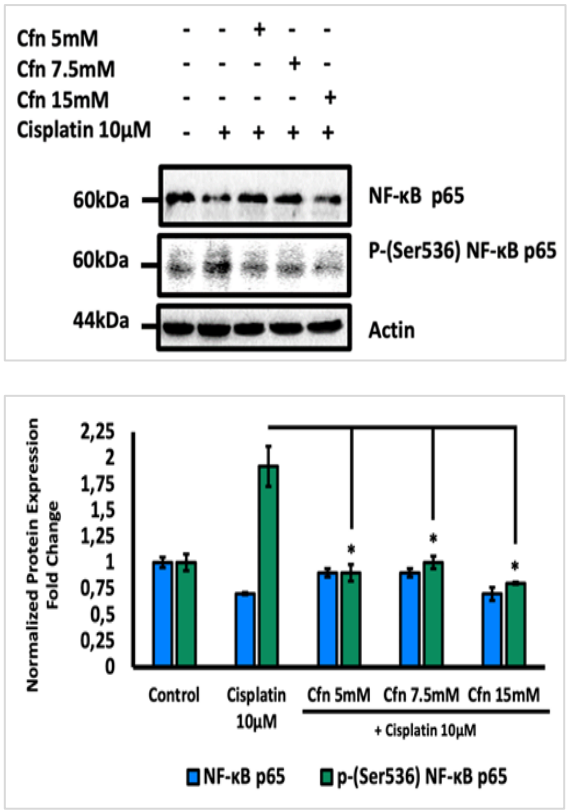

Figure 3.

The effect of caffeine on NF-κB signaling in prostate cancer cells. Cells were treated with vehicle, 10µM cisplatin or its combination with 5mM, 7.5mM and 15mM Cfn for 24 h and the levels of total NF-κB and phospho(Ser536) NF-κB protein levels were analyzed by immunoblotting studies. Beta-actin was used as a loading control. Changes in protein expressions were graphed in terms of fold increase. (n=3) (* p<0.05)

3.1.4 Caffeine synergistically enhances cisplatin-induced cell cycle arrest.

Induction of cell cycle arresting in cancer cells is a powerful approach for the prevention of carcinogenesis. For this aim, we tested the effect of the combined treatment of cisplatin and Cfn on cell cycle-related regulatory protein levels, including cyclin A2, cyclin B1 and cyclin E1. Our results indicated that cisplatin administration decreased cyclin B1 and cyclin E1, whereas the levels of cyclin A2 were not affected compared with the control group. Co-treatment of cisplatin and Cfn strongly decreased the protein levels of all tested cyclin proteins in a dose-dependent manner compared to the control or cisplatin-treated group (Figure 4).

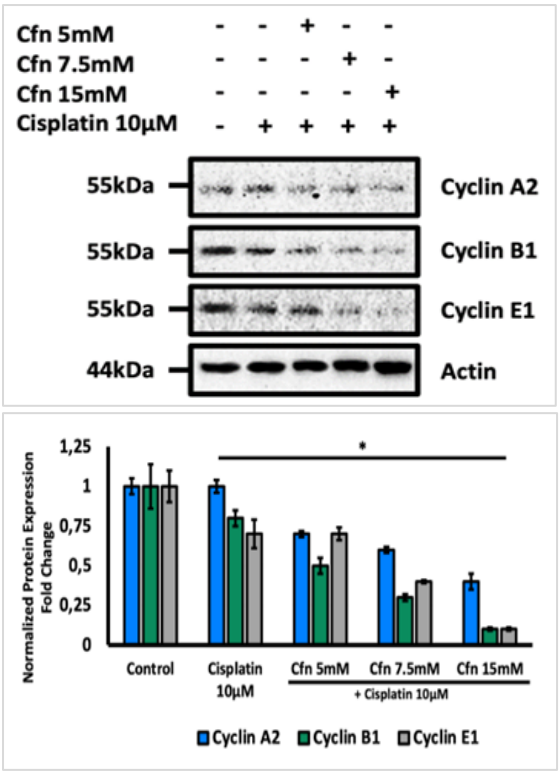

Figure 4.

Evaluation of the effect of caffeine on the level of cell cycle-related cyclin proteins in prostate cancer cells. Cells were treated with vehicle, 10µM cisplatin or its combination with 5mM, 7.5mM and 15mM Cfn for 24 h and the levels of cyclin A2, cyclin B1 and cyclin E1 were examined by immunoblotting studies. Beta-actin was used as a loading control. Changes in protein expressions were graphed in terms of fold increase. (n=3) (* $p<0.05$)

3.1.5 Co-treatment of Caffeine and cisplatin remarkably reduces androgenic and angiogenic signals in LNCaP cells.

To examine the possible improving anti-cancer effect of Cfn on cisplatin, we co-treated the LNCaP cells with cisplatin and Cfn and examined the AR, PSA and VEGF-A protein levels by immunoblotting assay. Cisplatin treatment markedly decreased the expression levels of VEGF-A and PSA, whereas AR levels had not affected compared to the control group. Co-administration of cisplatin and Cfn strongly reduced the AR, PSA and VEGF-A protein levels in a dose-dependent manner (Figure 5).

Testing the effects of caffeine on androgenic signaling in prostate cancer cells. Cells were treated with vehicle, 10µM cisplatin or its combination with 5mM, 7.5mM and 15mM Cfn for 24 h and then the expression level of VEGF-A, AR and PSA were analyzed by immunoblotting. Beta-actin was used as a loading control. Changes in protein expressions were graphed in terms of fold increase. (n=3) (* $p<0.05$)

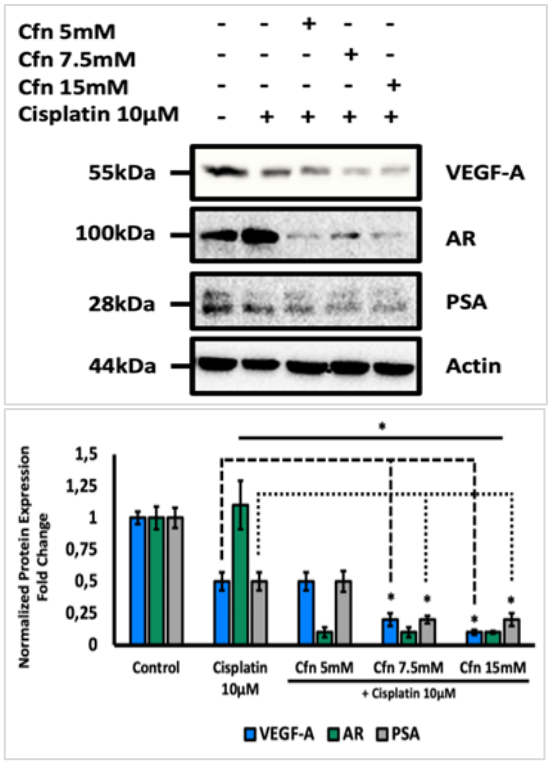

Figure 5.

3.2 Discussion

The estimated number of new cases in 2023 of PCa is expected to have the highest number of 288,300 among genital system-related cancer cases and about 34,700 people will be thought to die from PCa [35]. Current treatment options for PCa management are radiation, surgery, chemotherapy and androgen deprivation therapy [1-8]. Moreover, therapeutic agents, including, doxorubicin, paclitaxel, carboplatin, etoposide or combined therapies and antiandrogens are used to reduce symptoms and inhibit cancer growth [36,37]. However, these chemotherapeutics have exhibited a systemic toxic effects on organs that can cause significant side effects on human health in long-term use [15,36]. Therefore, there is a need for natural products that can expand the usage of chemotherapeutics by increasing their therapeutic efficacy. Studies demonstrated that coffee consumption diminished the risk of some types of cancer such as colorectal, skin, liver, lung and prostate as well [38-42].

Cfn is the most frequently consumed neuroactive natural product in the world and is a part of the daily diet of many people [43]. It has various beneficial effects, including anti-oxidant, anti-inflammatory and anti-cancer [16-17]. Although the effects of several derivatives of Cfn have been shown on PCa cells through inhibiting carcinogenic features such as proliferation and migration, the molecular mechanisms of action of Cfn have not been known yet [42].

Platinum-based-chemotherapeutic agent, cisplatin is best known for curing testicular cancer and is also

used in the treatment of other cancer types, including ovarian, bladder, head and neck, lung and cervical cancers [44]. Cisplatin exhibits cytotoxic or anti-neoplastic effects on human tumors. Although it is a broad-spectrum anti-cancer drug, it can lead to drug resistance clinically which produces many side effects [43-45]. Therefore, there is still a need to develop more effective treatment strategies to overcome the rapid progression of PCa and alleviate the various side effects that may result from chemotherapeutics.

In the present study, we examined the possible booster effect of Cfn on the therapeutic power of cisplatin in PCa. Firstly, we tested the effect of cisplatin and its combination with Cfn on cell viability of LNCaP cells. We found that combined treatment of cisplatin with Cfn significantly improved the anti-cancer effect of cisplatin in a dose-dependent manner (Figure 1). These results suggest that Cfn may have an enhancer effect on cisplatin's anticancer properties. Next, we evaluated the effect of cisplatin and its combination with Cfn on p38 MAPK signaling, our findings showed that cisplatin-induced p38 activation in LNCaP cells. However, combined treatment of cisplatin with Cfn markedly decreased the p38 MAPK activation by inhibiting the phosphorylation of p38 MAPK protein. p38 MAPK signaling has been associated with resistance to chemotherapeutics in several cancers [46,47]. Activation of p38 MAPK by cisplatin has been previously reported [48]. Also, it was reported that blockage of p38 MAPK potentiates cisplatin-induced apoptosis in growth-arrested kidney tubular epithelial cells [49]. Moreover, p38 MAPK inhibition improved the sensitivity of 5-fluorouracil (5-FU)-resistant SW480 (SW480/5-FU) human colon cancer cells to noscapine, an oral anti-tussive agent, through suppressing resistance [48]. Additionally, Pereira et al. reported that inhibition of p38 MAPK signaling sensitized the cisplatin-induced apoptosis in colon cancer cells and in a mouse model for breast cancer [50]. Consistent with the literature, our results suggest that Cfn increases the therapeutic efficacy of cisplatin in PCa cells (Figure 2).

NF-κB is a pivotal transcription factor that regulates inflammatory responses, apoptosis, cell proliferation and survival. Also, it has a role in cancer progression and chemoresistance mechanisms through the activation of a multitude of mediators, including anti-apoptotic genes [49,51]. Present data indicated that cisplatin treatment induced the NF-κB activation by inducing phosphorylation at Ser536 position. Moreover, co-treatment of cisplatin with Cfn markedly decreased the phosphorylated NF-κB levels (Figure 3). NF-κB activation has been associated with cisplatin resistance [52]. In a study conducted with non-small cell lung cancer cells, it was reported that suppression of NF-κB activation by the NF-κB small molecule inhibitor, DHMEQ, improved cisplatin sensitivity [53]. Our results suggest that Cfn increases the susceptibility of PCa cells to cisplatin by decreasing NF-κB activation. The cell cycle arrest effect of cisplatin on cancer cells is known [53,54]. Cyclin proteins activate the phase-specific cyclin-dependent kinases for progression through the cell cycle [55]. Cyclin B1 interacts with and activates the Cdk1 protein, allowing cells to enter the mitotic phase [56]. Cyclin E1 regulates the S-phase transition and DNA synthesis associated with mitosis through activating CDK2 [57]. Also, cyclin A2 is a crucial regulator protein of participation in the regulation of the S phase as well as the mitotic entry [58]. To test the enhancer effect of Cfn on cisplatin, we co-treated the LNCaP cells with Cfn and cisplatin. Our data indicated that Cfn strongly increased the cell cycle-arresting effect of cisplatin by decreasing the protein levels of cyclin A2, cyclin B1 and cyclin E1 (Figure 3). These results suggest that co-treatment of Cfn with cisplatin may strongly induce cell cycle arrest in LNCaP cells.

Lastly, we evaluated the augmenter effect of Cfn on cisplatin in PCa cells, for this aim we examined the androgenic signaling and angiogenic signal-related protein levels by immunoblotting. We found that the AR protein level, which is a ligand-activated transcription factor and an important mediator in PCa progression, remarkably decreased dose-dependently by co-administration of cisplatin and Cfn. Also, alone cisplatin treatment did not cause a prominent alteration in AR protein levels. Also, PSA levels decreased in a dose-dependent manner by co-administration of cisplatin and Cfn (Figure 5). Altered AR signaling is known to regulate the PCa progression-related features such as migration, invasion and differentiation [29]. Therefore, the pharmacological inhibition of AR signaling by AR signaling inhibitors (ARSIs) is being studied extensively [59,60]. These results suggest that Cfn can be used as a supplement in addition to traditional treatments as a natural product in the treatment of PCa. Additionally, we tested the effect of Cfn on VEGF-A levels, which is a crucial mediator for angiogenesis in cancer. It plays a pivotal role in tumor growth and dissemination [61,62]. Our data indicated that Cfn remarkably improved the weakening effect of cisplatin on VEGF-A expression in LNCaP cells (Figure 5).

Intrinsic or acquired resistance to chemotherapeutics is one of the biggest obstacles to cancer treatments. Herein, we evaluated the possible enhancer role of Cfn on the therapeutic efficiency of cisplatin in PCa cells. Present data suggest that Cfn administration may remarkably improve the chemotherapeutic efficiency of cisplatin by reducing the protein levels of cell cycle-related cyclin A2, B1 and E1 levels, decreasing the activation of NF-κB, p38 MAPK and AR signaling and reducing the VEGF-A levels (Figure 6).

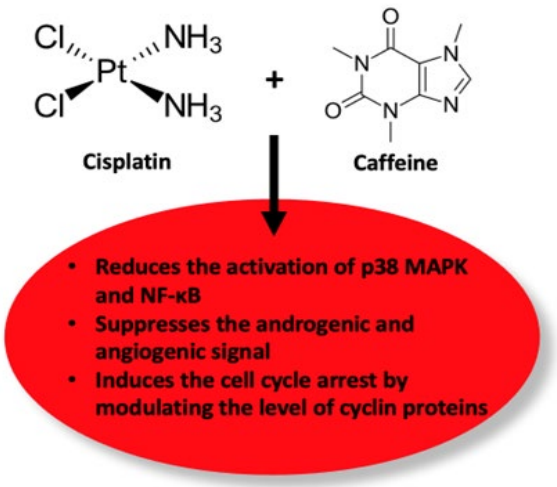

Figure 6. Mechanistic representation of co-treatment of cisplatin and caffeine on PCa cells.

4. Conclusion

Present data suggest that Cfn may be a potent enhancer for chemotherapeutics in PCa and it can improve treatment success by reducing the toxic dose of chemotherapeutics. However, further analysis is needed to confirm the booster effect of Cfn on cisplatin and also the synergistic mechanism of Cfn on cisplatin in PCa.

5. Acknowledgements and Disclosures

We thank Suleyman Demirel University-Innovative Technologies Application and Research Center.
The authors declare no competing financial interests.
The data generated in this study are available upon request from the corresponding author.
This study does not require any ethical permission.
This study was partially supported by Suleyman Demirel University internal funds (TSG-2021-8302, TAB-2020-8253).

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