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Original Article

Effects of betulinic acid on AKT/mTOR pathway in renal cell carcinoma

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ABSTRACT

Objective: Renal cancer is the most lethal among urological cancer. Treatments of renal cell carcinoma (RCC) may be possible by immune checkpoint inhibitors and drug treatment targeting different molecules. We aimed to determine the apoptotic effect of betulinic acid and its effects on expressions of apoptosis-associated genes AKT-1 and mTOR in RCC cells.

Material and methods: In this study, we investigated the apoptotic activity of betulinic acid in CAKI-2 cell line and its effect on AKT-1 and mTOR gene expression levels. In order to do so, following analyses were conducted: WST-1 to identify the toxic effect of betulinic acid, Caspase-3/BCA to detect caspase enzyme activity, Annexin-V and ELISA to determine for apoptotic effect, and finally, real-time PCR for expression levels of AKT-1 and mTOR.

Results: Our study showed that different concentrations of betulinic acid induced apoptosis in renal cancer; however, no effect was observed in healthy cells. In gene expression analysis, there was statistically significant decrease in AKT-1 expression level while increasing mTOR expression level.

Conclusion: We suggested that betulinic acid with its apoptotic effect on RCC line and nontoxic effect on healthy cell line and the effects on AKT/mTOR pathway may be a potential anticancer drug promising for future studies.

Keywords: Apoptosis; betulinic acid; expression; pathway; renal cancer.

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Introduction

Kidney cancer is the sixth and 10th most frequent cancer in man and woman, respectively.[1] Kidney cancer is not a single disease; it consists of a heterogeneous group of cancers with many different molecular and genetic alterations. This group may also have different histological features and clinical presentations.[2,3] The most common type of kidney cancer is renal cell carcinoma (RCC), which is 90% of all kidney cancer cases and occurs in renal tubular epithelial cells.[4] Among all RCC cases, the most common subtype is a clear cell renal cell carcinoma (ccRCC). In 2018, according to the GLOBOCAN research, 403,262 new kidney cancer cases were diagnosed, and 175,098 patients died of kidney cancer.[5]

As the treatment options of RCC, surgery and nephrectomy are used in the treatment of localized RCC patients, while systemic treatment is used in the treatment of metastatic RCC or patients who have recurrence after local treatment.[6] Systemic treatment is determined by the molecular biology of the RCC subtype which patients have. Systemic treatment options may include immune checkpoint inhibitors, mTOR inhibitors, and approved drugs targeting VEGF and VEGFR.[7]

The PI3K/AKT/mTOR pathway is one of the pathways with overexpression in various types of cancer including RCC.[8] AKT-1 and mTOR, a member of PI3K/AKT/mTOR pathway, are involved in regulation of cell growth, proliferation survival, and cellular metabolism and

homeostasis.[9] Therefore, any mutation that occurs in the components of PI3K/AKT/mTOR pathway may cause overactivation of AKT-1 and mTOR, leading to tumor progression.[10]

Triterpenoids are natural compounds of 30 carbons derived from various plants with more than 20,000 members.[11] Also these compounds are a group of secondary metabolites, capable of defense and repair against environmental stress and injury in plants.[12] Triterpenoids are divided into two main groups, tetracyclic triterpenes and pentacyclic triterpenes, and their main derivatives are lupane, oleanne, ursane, and cucurbitane.[13,14] Triterpenoids and its derivatives have wide range of biological effects such as anticancer,[15] anti-inflammatory,[16] antiviral,[17] and antibacterial[18] activities.[19]

Betulinic acid, a member of lupane type of pentacyclic triterpenoids, exhibits similar biological activities with other triterpenoids, particularly anticancer activities. In recent years, investigations of anticancer and cytotoxic activities of betulinic acid have shown that it enhances apoptosis throughout mitochondrial pathway,[20,21] and without toxic effect on healthy cells, it induces apoptosis in cancer cells.[22–24]

Taking into account all of these, the aim of our study is the examination of apoptotic activity of betulinic acid in RCC cells and determination of its effects on apoptosis-associated genes, AKT-1 and mTOR.

Material and Methods

Cell Culture

The CAKI-2 clear cell renal cell carcinoma (ccRCC) and MRC-5 lung fibroblast cell lines were acquired from American Type Culture Collection (ATCC, USA). The CAKI-2 (ATCC® HTB-47™) cell line was cultured in McCoy's 5A medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. The MRC-5 (ATCC® CCL-171™) cell line was cultured in EMEM medium supplemented with 1% penicillin-streptomycin and 10% FBS at 37°C in 5% CO_2.

Main Points

- Anticancer activity of betulinic acid in renal cancer cell line.
- Different concentrations of betulinic acid induced apoptosis in renal cancer.
- AKT/mTOR pathway may be a potential anticancer drug promising for future studies.

Reagents and Assay Kits

Cell culture reagents were purchased from Thermo Fisher Scientific (USA). Betulinic acid was purchased from Sigma–Aldrich (USA). WST-1 cell proliferation assay and Cell Death Detection ELISA kits were purchased from Roche Life Sciences (Germany). Annexin-V and Caspase-3 assay kits were purchased from Merck Millipore (USA) and BioVision (USA), respectively. The RNA isolation, cDNA synthesis, and qPCR assay kits were acquired from Jena Bioscience (Germany).

Cell Proliferation Assay

The cell viability effect of betulinic acid on cell lines was detected using WST-1 assay. CAKI-2 and MRC-5 cell lines were seeded at a density of 10^4 cells per well in 96-well plates. When cells adhered to wells after 24 hours incubation, they were treated with different concentration of betulinic acid (1, 2.5, 5, 7.5, 10, 25, and 50 μM) for 24, 48, and 72 hours. WST-1 colorimetric cell proliferation assay was performed after each incubation period. WST-1 solution (10 μL) was applied to each well, and the well plates were incubated for 4 hours at 37°C. Color development was measured at 460 nm using microplate ELISA reader (Thermo Fisher Scientific, Germany). Cell viability was determined in comparison to the untreated cells.

Caspase-3 Enzyme Activity Assay

Detection of changes in Caspase-3 enzyme activity is one of the important indicators of apoptosis. Thus, CAKI-2 and MRC-5 cells were seeded at a density of 5×10^5 cells per well in six-well plates. After 24 hours incubation, cells were treated with 25 and 50 μM betulinic acid or negative control. After incubation period, the cells were lysed by 100 μL cell lysis buffer and incubated on ice for 10 minutes before centrifugation. 50 μL of supernatant samples was mixed with 50 μL reaction buffer and 5 μL DEVD-pNA substrate and incubated for 2 hours. The reactions were read at 405 nm using microplate ELISA reader.

Detection of Apoptotic Nucleosomal DNA

Using Cell Death Detection ELISA kit, the presence of mono or oligonucleosomes in the cellular supernatant was examined, and thus, apoptotic cell death was measured. The reactions were read at 405 and 490 nm using microplate ELISA reader. The color development of samples was identified as an enrichment factor of DNA fragments (mono or oligonucleosomes), and the results were evaluated in comparison to untreated cells.

Flow Cytometry Analysis of Apoptosis

The phosphotidylserine, which is found on the inner surface of the cell membrane in normal cells, is translocated to the outer surface of the cell membrane in apoptotic cells. This cellular marker of apoptosis was detected by staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI)

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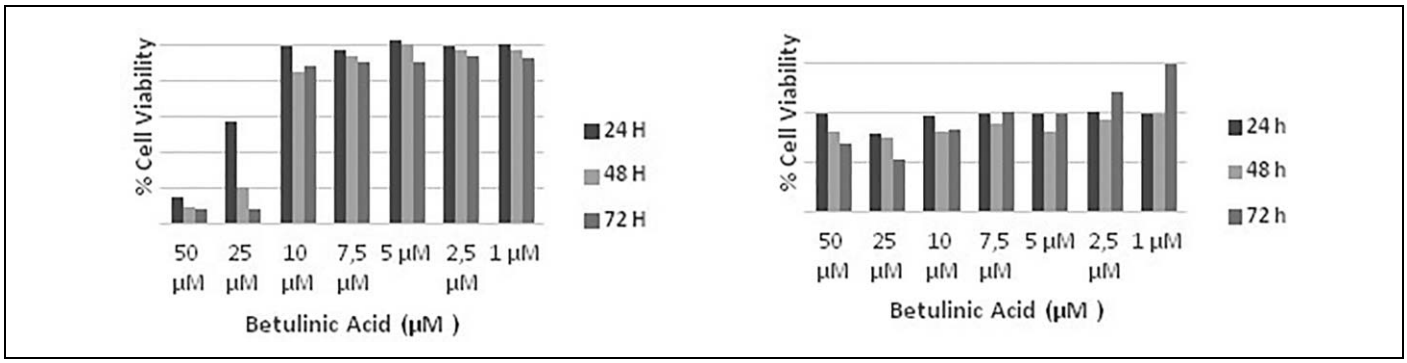

Figure 1. The effect of betulinic acid on cell viability and cytotoxicity. Effect of betulinic acid on the viability of CAKI-2 cells (a) and MRC-5 cells (b), depending on dose and time. The results are the means of two independent experiments ($P < .05$).

fluorescein dyes. For this analysis, cells were seeded at a density of 10^5 per well in six-well plates and were treated with different concentrations of betulinic acid (25 and 50 μM for 24 hours). At the end of 24 hours, cells were centrifuged and washed with PBS. Then, the cells were suspended with 1× binding buffer at a concentration of 1×10^6 cells mL^{-1}. Following this, 5 μL Annexin V-FITC and 5 μL PI were added to 100 μL of suspension cells. The cells were incubated for 20 minutes at room temperature in the dark. The results were analyzed using flow cytometry.

qPCR Gene Expression Analysis

After each cell line was treated with different concentrations of betulinic acid (25 and 50 μM for 24 hours), total RNA was extracted from cells, and cDNA synthesis was performed from RNA extractions. The qPCR analysis was performed using Bio-Rad. In order to detect fold changes in mRNA levels of genes (AKT-1 and mTOR), cycle threshold (C_T) values of each gene were subtracted from C_T values of housekeeping gene (GAPDH). $\Delta\Delta C_T$ values of genes were calculated via subtracting ΔC_T of control from ΔC_T of target genes. The fold change was calculated as $2^{-\Delta\Delta CT}$.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM SPSS Corp.; Armonk, NY, USA). The significance limit was accepted as $P < .05$. The Mann–Whitney U test was used for intergroup evaluation.

Results

Betulinic Acid Decreased Cell Viability in Renal Carcinoma Cells

We investigated the cytotoxic effects of betulinic acid on CAKI-2 and MRC-5 cell lines using the WST-1 colorimetric cell proliferation assay. The cells were seeded and incubated with increasing concentration (1-50 μM) of betulinic acid for 24, 48, and 72 hours. WST-1 assay was performed at the end of each incubation period. The results showed that cell viability of CAKI-2 cells treated with 25 and 50 μM betulinic acid decreased significantly (Figure 1). However, it was observed that betulinic acid treatment had no significant effect on cell viability of MRC-5 cells.

Betulinic Acid Increased Caspase-3 Enzyme Activity

To determine caspase-3 enzyme activity, which is one of the apoptotic indicators, cells were incubated with 25 and 50 μM betulinic acid for 24 hours. The results showed that in CAKI-2 cell line, 1.4-fold increase as a result of 25 μM treatment and 1.7-fold increase as a result of 50 μM treatment in caspase-3 enzyme activity were observed (Figure 2).

Betulinic Acid Induced Apoptosis in Renal Carcinoma Cells

In order to detect apoptosis and necrosis in betulinic acid treated cells, we performed Annexin V assay. In CAKI-2 cell line, apoptotic cell death was observed 7.5% at 25 μM and 14.25% at 50 μM doses of betulinic acid for 24 hours incubation period ($P < .05$). In the healthy cell line MRC-5, it was observed that betulinic acid treatment had no effect on apoptosis of cells (Figure 3).

Betulinic Acid Increased Apoptotic Nucleosomal Enrichment Factor

In order to determine the apoptotic activity of betulinic acid on CAKI-2 and MRC-5 cell lines, changes in nucleosomal enrichment factor were determined by treating betulinic acid at 25 and 50 μM concentration for 24 hours. 25 and 50 μM betulinic acid treatment on CAKI-2 cell line was resulted in 2.33- and 2.83-fold increases in enrichment factor, respectively, compared to healthy MRC-5 cell line (Figure 4, $P < .05$).

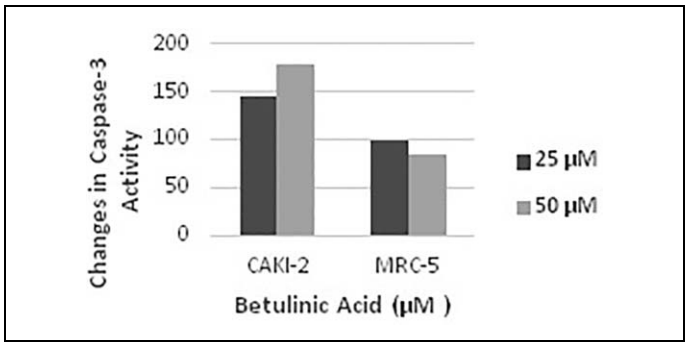

Figure 2. The effect of increasing concentration of betulinic acid on caspase-3 enzyme activity in CAKI-2 and MRC-5 cells. These results are the means of two independent experiments ($P < .05$).

Betulinic Acid Changed AKT-1 and mTOR Gene Expression Profiles

It is known that AKT-1 and mTOR induce cell survival. Using the real-time PCR analysis, we determined the effect of betulinic acid on AKT-1 and mTOR gene expression levels. In CAKI-2 cells treated with 25 μM betulinic acid, 0.41-fold change in AKT-1 expression ($P = .034$) and 1.61-fold change in mTOR gene expression ($P = .037$) were observed. On the other hand, in 50 μM betulinic acid treatment, 0.38-fold change in AKT-1 gene expression ($P = .037$) and 1.94-fold change in mTOR gene expression were detected (Figure 5). These results demonstrated that betulinic acid caused a statistically significant decrease in AKT-1 gene expression while increasing mTOR gene expression.

Discussion

Betulinic acid is a lupane-type pentacyclic triterpenoid, which consists of six isoprene units, and is isolated from various plants.[25] Betulinic acid performs its anticancer activity, which is one of the biological activities, mainly through the mitochondrial pathway.[26]

In recent years, many studies have been conducted to identify the anticancer activity of betulinic acid. In colorectal cancer cell lines, HCT116, SW480, and DLD-1, betulinic acid reduced cell viability as it increased the number of apoptotic cells in time- and dose-dependent manner. Furthermore, it was determined that betulinic acid increased the amount of Bax and Caspase-3 protein levels and decreased the amount of Bcl-2 protein level and triggered the loss of mitochondrial membrane potential. Thus, it was found that betulinic acid induces apoptosis by affecting the mitochondrial pathway.[24] Similarly, in hepatocellular carcinoma cell line HepG2, betulinic acid induced cell death. The effect of betulinic acid on apoptosis via mitochondrial pathway was investigated by studies on antiapoptotic, proapoptotic Bcl-2, and Caspase-3 protein levels. Betulinic acid increased proapoptotic Bcl-2 and Caspase-3 protein levels while decreasing antiapoptotic Bcl-2 protein levels.[23] On the other hand, Lee et al[22] extracted potential anticancer compounds including betulinic acid, from *Cornus walteri*. In ovarian cancer cell line A2780, among other compounds, betulinic acid showed highest toxic effect on cells. Annexin-V analysis showed that betulinic acid induced cell apoptosis in dose-dependent manner. Finally, betulinic acid enhanced apoptosis by modulating Bcl-2 protein levels.

In addition to these studies, betulinic acid affects different molecules and pathways in apoptosis apart from its effects to mitochondrial pathway. Shankar et al[27] found that in prostate cancer cells, betulinic acid triggered apoptosis by modulating Bax/Bcl-2 and decreased expressions of p-IKKα and IkBα, which causes NF-κB to direct nucleus. Xu et al[28] investigated the effects of betulinic acid in cervical cancer cells HeLa and

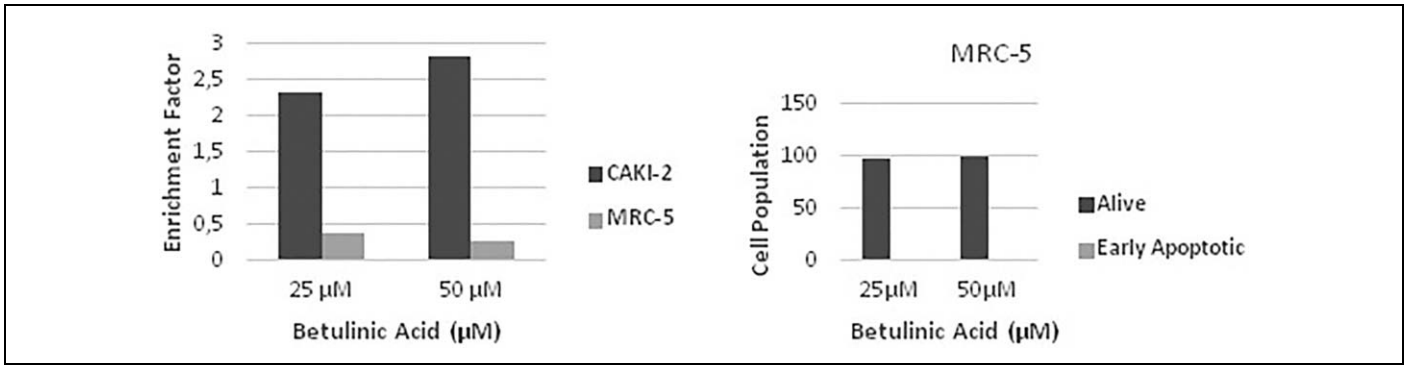

Figure 3. The effect of betulinic acid on cell apoptosis. Using flow cytometry analysis, the effect of betulinic acid on apoptosis of CAKI-2 (a) and MRC-5 (b) cells when applied specified dose (25 and 50 μM) was showed. These results showed in (a) and (b) are means of two independent experiments ($P < .05$).

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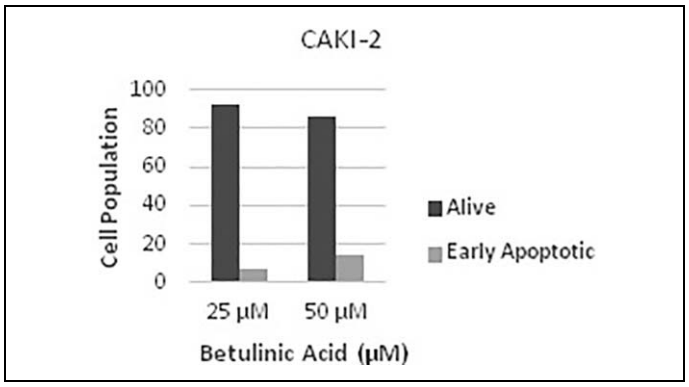

Figure 4. The effects of betulinic acid on nucleosomal enrichment factors. The detection of nucleosomal enrichment factor as a result of increasing concentration of betulinic acid in CAKI-2 and MRC-5 cells. These results are means of two independent experiments ($P < .05$).

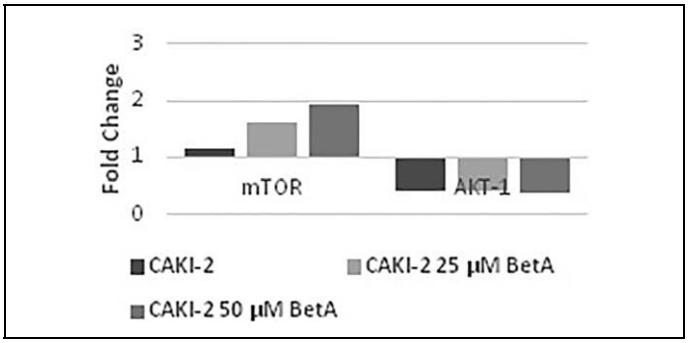

Figure 5. Betulinic acid changed AKT-1 and mTOR gene expression levels in renal cell carcinoma. The analysis showed that betulinic acid caused a statistically significant decrease in AKT-1 gene expression while increasing mTOR gene expression ($P < .05$).

found that betulinic acid treatment caused significant decrease in PI3K (p110a), PI3K (p85), p-AKT (Ser473), and p-AKT (Thr308) in time- and dose-dependent manner. In another investigation in human multiple myeloma cells, Pandey et al[29] showed that betulinic acid decreased STAT3 phosphorylation by increased SHP-1 expression, the negative regulatory of JAK/STAT pathway. Besides, it also reduced Bcl-2, Bcl-xL, Survivin, and Cyclin D1 expressions.

In this study, we determined apoptotic effect of betulinic acid on RCC cell line CAKI-2. We executed WST-1, Caspase-3 enzyme activity, Annexin-V, ELISA, and real-time PCR to determine the effect of betulinic acid on CAKI-2 cells viability, cellular apoptosis, and AKT/mTOR gene expression levels in time- and dose-dependent manner. According to our results in WST-1 and Annexin-V analysis, betulinic acid reduced cell viability and induced cell apoptosis. The analysis of Caspase-3 enzyme activity and ELISA nucleosomal enrichment factor suggested the apoptotic activity of betulinic acid. In addition, we demonstrated that betulinic acid significantly inhibited AKT-1 gene expression. These results correlate with the studies described earlier.

There are some limitations in this study. Another renal cell line is needed to examine and to determine AKT-1 and mTOR levels using another technique, such as Western blot analysis.

In conclusion, these data showed that apoptotic activity of betulinic acid and its potential impact on AKT/mTOR pathway suggest that betulinic acid may be a potential anticancer drug. Future studies will help to understand the molecular mechanism of anticancer effects of betulinic acid on this pathway.

Ethics Committee Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - E.S.İ., A.E.; Supervision - A.E.; Data Collection and/or Processing - M.N.A., B.E.; Analysis and/or Interpretation - A.E.; Critical Review - B.Ç.

Conflict of Interest: The authors have no conflicts of interest to declare.

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