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ORIGINAL ARTICLE

Juglone Effects by Dual Way on mTOR Gene Expression, Which Plays Central Role in Cell Growth, Survival and Metabolism, in PANC-1 and BxPC-3 Pancreatic Cancer Cell Lines

Juglon, PANC-1 ve BxPC-3 Pankreas Kanser Hücre Hatlarında Hücre Büyümesi, Hayatta Kalma ve Metabolizmada Merkezi Rol Oynayan mTOR Gen İfadesine İki Yönlü Etki Eder

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ABSTRACT

Background/Aims: Juglone, as a naphthoquinone, has been shown to have cytotoxic and apoptotic effects in various cancer cells and besides this effects it was reported to have anti-invasive and anti-metastatic effects in PANC-1 and BxPC-3 cells in our previous studies. In this study, we investigated the effects of juglone on *GRP75, TFAM* and *mTOR* genes encoding key proteins associated with mitochondrial biogenesis and activation in PANC-1 and BxPC-3 pancreatic cancer cells since mitochondria have central roles in cancer cell survival, metastasis and therapeutic resistance.
Methods: In our study; 5, 10, 15 and 20 μM juglone doses were selected as the application doses considering the IC50 value determined after MTT test results and the expressions of the target genes were analyzed by qPCR method after application of juglone doses for 24 hours.
Results: Our study results revealed that juglone had an opposite and strong effects on *mTOR* expression in both cell lines.
Conclusion: Our findings suggest that juglone has a developable potential and is a promising theurapeutic agent to develop new strategies for the battle with cancer with those effects on *mTOR* gene which plays a central role in cellular homeostasis and several cellular events including cell growth, survival and metabolism.

Key words: Juglone, Pancreatic cancer, Mitochondrial biogenesis, mTOR gene expression

ÖZ

Arkaplan/Hedefler: Bir naftakinon olarak juglonun çeşitli kanser hücrelerinde sitotoksik ve apoptotik etkisi gösterilmiş, daha önceki araştırmalarımızda PANC-1 ve BxPC-3 hücrelerinde bu etkilerinin yanı sıra anti-invaziv ve anti-metastatik etki gösterdiği rapor edilmiştir. Bu çalışmada kanser hücrelerinin sağkalımı, metastaz ve tedaviye direnç gelişmesi süreçlerinde merkezi rolü olan mitokondrial biyogenez ve aktivasyon ile ilişkili anahtar proteinleri kodlayan *GRP75, TFAM* ve *mTOR* genleri üzerine juglonun etkileri PANC-1 ve BxPC-3 pankreas kanseri hücre hatlarında araştırılmıştır.
Metodlar: Çalışmamızda MTT testi sonuçlarından elde edilen IC50 değeri dikkate alınarak 5, 10, 15 ve 20 μM juglon dozları uygulama dozu olarak seçilmiş ve juglonun belirlenen dozlardaki 24 saatlik uygulamasından sonra hedef genlerin ifadeleri qPCR yöntemiyle incelenmiştir.
Bulgular: Çalışma sonuçlarımız, juglonun her iki hücre hattında da *mTOR* ekspresyonu üzerinde zıt ve güçlü bir etkiye sahip olduğunu ortaya koydu.
Sonuç: Çalışma bulgularımız, hücresel homeostazda, hücre büyümesi, hayatta kalması ve metabolizması dahil olmak üzere bir dizi hücresel olayda merkezi rol oynayan *mTOR gen* ifadesi üzerindeki bu etkileri ile juglonun geliştirilebilir bir potansiyele sahip olduğu ve pankreas kanseri ile savaşta yeni stratejiler için umut vaad eden bir terapotik ajan olduğu düşüncemizi desteklemektedir.

Anahtar Kelimeler: Juglon, Pankreatik kanser, Mitokondrial biyogenez, mTOR gen ifadesi

Introduction

Pancreatic cancer, one of the most aggressive malignancies worldwide, is associated with the biological aggressiveness of the tumor, high resistance to chemotherapeutic agents, and low survival rates (1, 2). Moreover, pancreatic cancer usually progresses without symptoms and it is diagnosed at advanced stages when vascular invasion and metastasis has developed. Overall, only 20% of patients are eligible for surgery at the time of diagnosis, and 80% of these suffer a fatal recurrence after surgery (3). The overall prognosis is extremely poor and survival is expected to be less than 1 year. Therefore, pancreatic cancer is currently estimated to represent the 7th cause of cancer-related death worldwide (4). The most common form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) occurring in approximately 90% of cases. PDAC develops in pancreatic ducts that perform the main exocrine functions of the pancreas (5). Despite several researches on chemotherapy, only minor improvements have been seen in survival over the past decade because of drug resistance as one of a major reason (6). Thus, new approaches for pancreatic cancer treatment are sorely needed to improve prognosis, overcome drug resistance and increase current survival rates.

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Juglone (5-hdroxy-1,4-napthoquinone), is a naphthoquinone found in the leaves, roots and bark of many walnut tree species including Juglans nigra (black walnut) and Juglans regia (English or Persian walnut) (7). It has been shown that juglone, known as having cytotoxic effects in various cancer cell lines (8, 9), induces mitochondrial-mediated apoptosis by regulating cell redox homeostasis especially through reactive oxygen species (ROS) (10).

Mitochondria, called as the power-house of the cell due to its main role in ATP production, also undertake many important functions such as ROS production, regulation of cell signaling, cell death and biosynthesis events. Because of these multiple functions in physiological conditions, mitochondria are important cellular stress sensors and give the cell flexibility for its adaptation to the environment. This flexibility enables the cell to survive in stress situations such as nutrient shortage and hypoxia. Therefore, mitochondria are key players in carcinogenesis. They support cancer cell survival, progression and metastasis, and even development of resistance to treatment by enabling the cancer cell to adapt its harsh environmental conditions, rearranging the metabolism and all related events (11, 12).

Because of these important and critical properties, mitochondria are important therapeutic targets not only for inducing apoptosis but also for stopping the development and metastasis of cancer cells by changing mitochondrial biogenesis and activation functions. Thus, in our study, we aimed to investigate the effects of juglone on the expression levels of Glucose Regulated Protein 75 *(GRP75)*, Mitochondrial Transcription Factor A *(TFAM)* and Mammalian Target of Rapamycin *(mTOR)* genes, which are the key molecules in mitochondrial biogenesis and activation in PANC-1 and BxPC-3 human pancreatic cancer cell lines.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines PANC-1 and BxPC-3 were provided from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured as indicated in our previous study (13, 14).

Application of juglone on PANC-1 and BxPC-3 cells

Juglone application doses were determined by considering the IC50 values as 21.25 for the PANC-1 cell line and 21.05 for BxPC-3 cell line, according to MTT analysis in our previous study (13, 14). The determined doses were applied for 24 hours as 5 µM, 10 µM, 15 µM and 20 µM juglone. Juglone was supplied commercially (Sigma-Aldrich Chemical Company, USA).

Gene expression analyzes

Total RNA isolation from PANC-1 and BxPC-3 pancreatic cancer cells was performed using TRIzol reagent. RNA quantification was done by spectrophotometer (NanoDrop, Thermo Fisher Scientific, USA). cDNA synthesis was carried out using RT-PCR kit (RTPL12®, Vivantis, Malaysia). The expression levels of target genes *GRP75, TFAM* and *mTOR*, were analyzed by quantitative real-time PCR (qPCR) technique. The primers used in the analysis are shown in Table 1 (15-18). β-actin was used as reference gene for normalization. PCR reaction, performed using the Roche Light Cycler96, and were set as follows; an initial denaturation at 95°C for 5 min was followed by denaturation at 95°C for 30 sec recurrently at 40 cycles, annealing at 60°C for 30 sec, and elongation at 72 °C for 30 sec. $2^{-\Delta\Delta CT}$ method was applied to analyze the relative changes in the gene expressions. In the interpretation of qPCR results, 2 fold increase and decrease were evaluated as significant depending on the general approach in the literature.

Table 1. Primers for qPCR analysis of gene expression.

Gene	Primer sequence (F, R)*	References
GRP75	F: 5'-GCTGTCAATCCTGATGAGGCTGTG-3'	(15)
	R: 5'-CTGGCTCTTCTTGGTTGGAATAGTGG-3'	
mTOR	F: 5'-AGTGGACCAGTGGAAACAGG-3'	(16)
	R: 5'-TTCAGCGATGTCTTGTGAGG-3'	
TFAM	F: 5'-AGCTCAGAACCCAGATGC-3'	(17)
	R: 5'-CCACTCCGCCCTATAAGC-3'	
β-*aktin*	F: 5'-ACTCTTCCAGCCTTCCTTC-3'	(18)
	R: 5'-ATCTCCTTCTGCATCCTGTC-3'	

*F: Forward Primer, R: Reverse Primer

Results

Cell culture

In our study, human pancreatic cancer cell lines PANC-1 and BxPC-3 were used. During the culture, the cells were observed under an inverted microscope and it was determined that the cells maintained their specific morphology and behavior (Figure 1).

Gene expression analyzes

GRP75 gene expression:

The expression level of target gene *GRP75* in PANC-1 cells decreased as 1.18 fold, 0.17 fold and 2.16 fold at 5 µM, 10 µM and 15 µM doses, respectively, compared to the control group after 24 hours of juglone application. Significant reduction was detected at only 15µM dose. And, 0.08 fold non significant increase was observed after 20 µM juglone application (Figure 2a).

After 24 hours of juglone treatment in BxPC-3 cell line, the expression level of *GRP75*, compared to the control group, was detected as 0.57 and 0.11 fold decrease at 5 µM and 15 µM, and 0.52 and 0.38 fold increase at 10 µM and 20 µM, respectively (Figure 2a). The changes were not significant.

***TFAM* gene expression:**

At the end of 24-hour juglone application in PANC-1 cell line, *TFAM* gene expression levels at 5 µM and 10 µM juglone concentrations decreased 0.37 fold and 3.91 fold, respectively, compared to the control group. A significant increase of 2.62 and 4.23 folds was observed at 15 µM and 20 µM juglone applications, respectively (Figure 2b).

The change of *TFAM* gene expression in BxPC-3 cell line compared to the control group after 24 hours of juglone application was detected as 0.56 and 0.46 fold increase at 5 µM and 15 µM, respectively. At 10 and 20 µM doses, decreases of 0.38 and 0.5 fold were recorded (Figure 2b). Changes were not significant.

***mTOR* gene expression:**

After 24 hours of 5 µM juglone application, the expression level of the *mTOR* gene in PANC-1 cells decreased 0.22 fold compared to the control group. On other hand, significant increases of 5.24 fold, 5.07 fold, and 3.38 fold at 10 µM, 15 µM and 20 µM juglone applications were detected (Figure 2c).

The expression level of the *mTOR* gene in BxPC-3 pancreatic cancer cells strikingly decreased 11.09 fold at 20 µM juglone concentration, while 1.57, 1.97 and 1.26 folds increases was recorded at by 5 µM, 10 µM, 15 µM juglone doses respectively (Figure 2c).

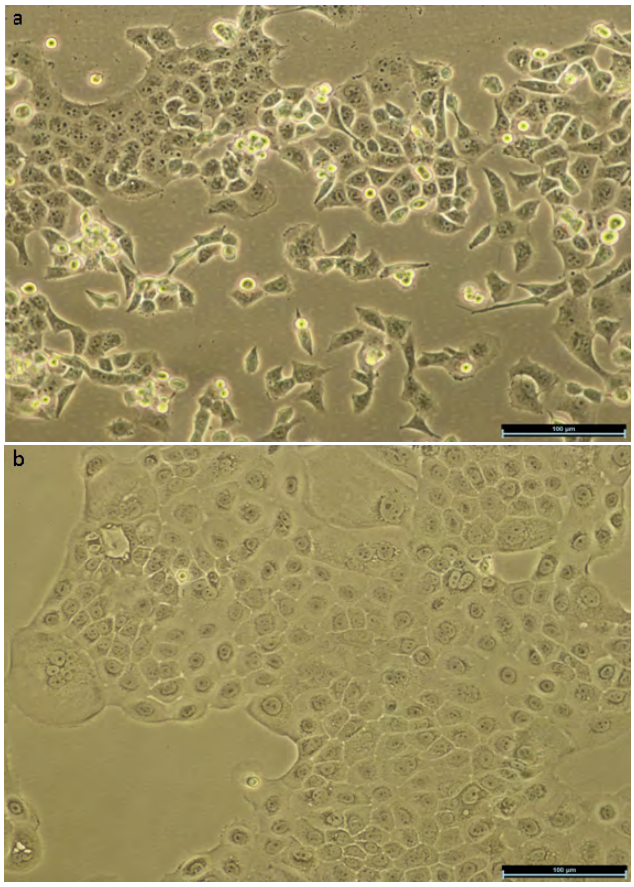

Figure 1. Visualization of a) PANC-1 cells and b) BxPC-3 cells under an inverted microscope (X10 magnification).

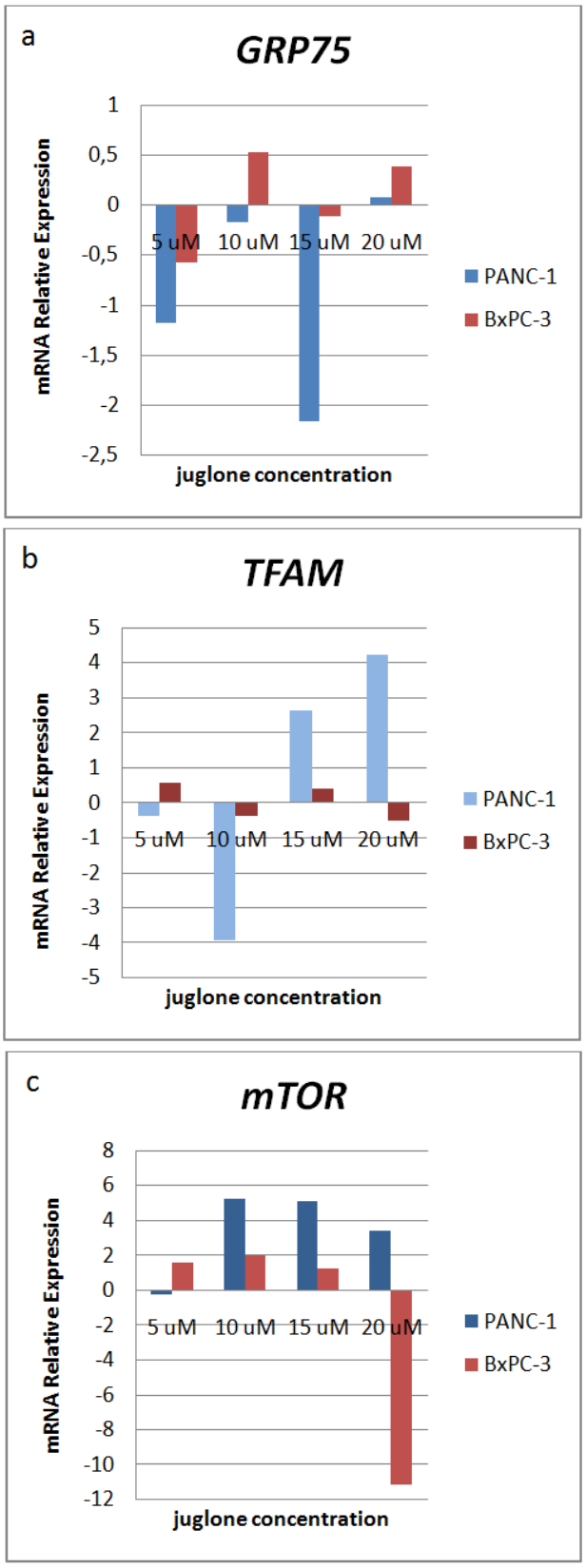

Figure 2. Effects of juglone on expression of a) *GRP75, b) TFAM,* and c) *mTOR* genes in PANC-1 and BxPC-3 cells for 24 h determined by the qPCR analysis.

Discussion

In our study, the effects of juglone, known as a secondary metabolite naphthoquinone compound, on the expression levels of *GRP75, TFAM* and *mTOR* genes, which are the key genes related to mitochondrial activation and biogenesis, were investigated in PANC-1 and BxPC-3 human pancreatic cancer cell lines. Our study results revealed that juglone had an opposite and strong effect on mTOR expression in both cell lines.

Mitochondrion is a dynamic organelle in which multiple functions are carried out, involved in the regulation of the functioning of intracellular signaling cascades, production of reactive oxygen species, fatty acid β-oxidation, amino acid metabolism, pyrimidine synthesis, phospholipid modifications, calcium regulation, cell survival, aging and death (19). It also has an important role in cell survival with its ability to adapt these multiple functions for cellular demands, environmental conditions and changing accessible substrates (20).

The maintenance of mitochondrial homeostasis is ensured by fine coordination between two opposing processes, namely the formation of new mitochondria by mitochondrial biogenesis and the removal of damaged mitochondria by mitophagy (21, 22). Fission is a self-renewal pathway in which two or more mitochondria are produced from already existing mitochondria. Conversely, mitochondrial fusion is defined by the union of several neighboring mitochondria (23). Both processes require highly regulated mechanisms to allow the formation of new and functional organelles and prevent loss of intramitochondrial content. Increased fusion or reduced fission promotes the formation of long mitochondrial networks, while increased fission or decreased fusion causes mitochondrial fragmentation. Fission and fusion machinery proteins also regulate intrinsic apoptotic pathways (24).

The Ca^{2+} level and the AMP/ATP ratio are the main stimulators of mitochondrial biogenesis. Increased Ca^{2+} and AMP levels directly or via CREB activate the peroxisome-proliferator-activated γ co-activator-la (PGC-1a), the master regulator of mitochondrial biogenesis (19). In addition, the increased NAD+/NADH ratio also causes PGC-1a activation. Activated PGC-la promotes expression of various mitochondrial proteins including TFAM, ultimately resulting in increased mitochondrial biogenesis (25).

GRP75 has been identified as a member of the heat shock protein 70 (HSP70) chaperone family based on sequence similarity although it is not a heat-activated protein (26). GRP75 (mortalin/PBP74/mthsp70) resides at the ER-mitochondria interface, called the mitochondria-associated membrane (MAM), at docking sites between two organelles that control the balance between cellular survival and death (27). A number of proteins are known to stabilize ER-mitochondria contact in MAM (28). Contact at this interface controls the exchange of metabolites, energy production, maintenance of mitochondrial shape, and more importantly, apoptosis. In MAM, inositol 1,4,5-trisphosphate receptors (IP3Rs), which are the calcium channels in the ER membrane and voltage-dependent anion channels (VDACs) in the outer mitochondrial membrane, are physically connected via GRP75. GRP75 is considered as a key protein that modulates ER-mitochondrial Ca^{2+} signaling via the IP3R-GRP75-VDAC1 complex and regulates the direct transfer of Ca^{2+} from the ER to the mitochondria (29). Calcium is one of the most important regulators of mitochondrial energy production and cell death. Regulation of *GRP75* expression and that ER-mitochondrial Ca^{2+} crosstalk in MAM is a new protective approach in cell death and anti-cancer therapy paradigms.

Ca influx into mitochondria has been shown to be determinative for tumor growth and metastatic behavior (30, 31). It has also been shown that decreased mitochondrial Ca^{2+} uptake has a role in the escape of cancer cells from apoptosis and as a result of *GRP75* gene silencing, it protects against cell death by preventing mitochondrial Ca^{2+} overload under cellular stress conditions (32). In addition, GRP75 binds to many proteins, including the tumor suppressor p53 (33).

In our study, it was observed that there was a 2.16 fold significant decrease in *GRP75* expression level after 15 μM dose of juglone application in PANC-1 cell line. On the other hand, in BxPC-3 cell line, no significant change was detected at all doses. Our results show that there is no significant effect of juglone on *GRP75* gene expression. Juglone is known to promote ROS-mediated apoptosis. Consistently, in our previous studies, it was determined that juglone induced apoptosis at increasing dose-dependently at all doses. However, in this study we consider that GRP75 has not a role in the apoptosis-inducing effect of juglone.

TFAM, also known as a transcription factor for mtDNA, belongs to a superfamily of HMG protein and is a multifunctional protein. TFAM plays a role in the packaging of mtDNA into a nucleoid-like structure, as well as in mtDNA replication and repair of damaged mtDNA (34). In a study, it was shown that the upregulation of TFAM expression in KB human epidermoid cancer cells and the changes in damage recognition activity contributed to the cancerization process of cells by avoiding cisplatin-induced apoptosis (35). In another study in lung cancer cell lines, it was reported that downregulation of TFAM caused cell cycle arrest in the G1 phase and significantly inhibited cell growth and migration through the activation of ROS-induced c-Jun amino-terminal kinase (36). In a study conducted with samples taken from patients with pancreatic adenocarcinoma, it was reported that *TFAM* gene expression worsened the clinical course of patients with PDAC by allowing PDAC progression through inhibition of apoptosis. TFAM was evaluated as an independent marker for poor prognosis in patients with PDAC after pancreatectomy (37). In line with the aforementioned studies, it can be interpreted that high expression of the *TFAM* gene contributes to the

development of cancer increasing cell proliferation and decreasing the proliferation of cancer cells with the decrease of *TFAM* gene expression.

When *TFAM* expression levels after juglone applications were evaluated in our study, a 3.91 fold decrease was observed at 10 µM in PANC-1 cell line, while an increase of 2.62 and 4.23 fold detected in 15 µM and 20 µM doses. No significant effect of juglone on TFAM gene expression levels was detected in BxPC-3 cell line. The fact that the result in PANC-1 cells is in different directions shows that it causes different responses according to the application doses. The increase in 15 and 20 µM doses suggests that juglone may affect different mechanisms by potentiating the increase in mitochondrial activation.

mTOR is a serine/threonine kinase that acts through two structurally and functionally different protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), to sense and integrate multiple intracellular and environmental signals (38). mTORC1 modulates various downstream signaling effectors that affect cell proliferation, survival and angiogenesis. mTORC1 also regulates translation, ribosome biogenesis, metabolism, and cellular response to hypoxia and autophagy (39). The regulatory function of mTORC1 in mitochondrial metabolism has also been demonstrated (40). On the other hand, mTORC2 signaling is involved in the organization of the actin cytoskeleton and cell migration (41). In Desai et al. (42)'s study on human T cells, the interaction of mTOR with mitochondrial elements in the subcellular fractions of cells and its connection specifically with the outer mitochondrial membrane were demonstrated. There is also evidence that mTORC1 functions as a regulator of fission and fusion dynamics through Metal Regulatory Transcription Factor 1 (Mtf1) translation (43). In studies conducted in various cancer, it has been shown that under radiation stress, mTOR passes to mitochondria and shifts the bioenergy pathway from aerobic glycolysis to oxidative phosphorylation, which is associated with increased resistance to radiotherapy (44, 45). Sustaining malignant phenotypes such as radio/chemotherapy resistance, tumor invasion, and metastasis, tumor cells acquired a metabolic plasticity, and mitochondria are at the center of this dynamic (46).

Increasing evidence is identifying activation of mTOR signaling as a common event in human cancers (47). Furthermore, mTOR is closely intertwined with the PI3K/AKT pathway, which is strongly associated with neoplastic disease (47). These properties have made mTOR an attractive option for the development of molecular targeted therapies. Hassan et al. (48) propose the development of mTORC1/2 inhibitor-based therapies in murine and human PDAC models to elucidate the role of mTOR as a therapeutic target in PDAC. Irianan et al. (49) demonstrated that inhibition of mTOR in KRAS-dependent PDAC subtypes leads to inhibition of tumorigenesis in vitro and in vivo. However, initial clinical studies in individuals with specific KRAS-associated mutations in molecularly defined PDAC subtypes did not obtain very effective results. However, studies showed that combined inhibition of multiple steps among the mTOR signaling pathway can lead to sustained responses by targeting tumor resistance mechanisms. The results of advanced late phase studies involving combined mTOR pathway inhibition are expected to be effective (49).

In our study, after 10, 15 and 20 µM applications of juglone on PANC-1 cells, mTOR gene expressions increased strongly 5.24, 5.07 and 3.38 fold, respectively. Strikingly, there was a very sharp decrease (approximately 11 fold) at 20 µM juglone application on BxPC-3 cells while there were moderate increases at other application doses. These different cell responses are possibly because of the fact that the two cell lines originate from different origins and thus varied molecular mechanisms due to the diverse driver mutations in both cells acts on distinct ways. Similarly, the observation in the literature that fisetin, a natural flavonoid applied to PANC-1 cells, reduces cell proliferation, but that the activation of the AMP-activated protein kinase/Mammalian Target of Rapamycin (AMPK/mTOR) signaling pathway increases after fisetin administration also supports our findings in PANC-1 cells (50).

The majority of studies in the literature envision mTOR inhibition as a therapeutic strategy in targeting cancer metabolism. In contrast, some studies describe overexpression of mTOR as a double-edged sword that triggers resistance to chemotherapy in cancer, on the other hand, it leads to metabolic vulnerability (51). It is thought that cancer cells can be effectively treated if the metabolic vulnerability created by the high expression of *mTOR* is targeted in cancer cells (51). In our study, we suggest that juglone may have targeted this metabolic deficit by further increasing mTOR gene expression in PANC-1 cells.

Our study findings propose that juglone can suppress cancer development through overactivation instead of inhibition of *mTOR* expression in KRAS-dependent PANC-1 cells, by being targeted the mTOR mediated metabolic vulnerability as mentioned above. Our previous findings that juglone suppresses proliferation, adhesion and invasion in PANC-1 pancreatic cancer cells and exerts a strong anticancer effect by inducing apoptosis support this proposition (13, 14).

On the other hand, our study results revealed that juglone has potent inhibitory effects leading a dramatically decrease as approximately 11 fold on KRAS nondependent BxPC-3 cells. We have already demonstrated the anticancer effects of juglone on BxPC-3 cells in our previous studies. Based on the anticancer effect of juglone in BxPC-3 cells showed in our previous studies, we propose that mTOR inhibition by juglone can prevent tumor growth in the treatment of the KRAS nondependent PDAC subtype.

The strong but opposite effects of juglone on *mTOR* gene expression in both cell lines supports the fact that the molecular identity and type of the cancer cell is very important for drug development and treatment

approaches. However, this argument needs to be investigated with more comprehensive studies.

Conclusion

In our study, the effects of juglone, known as a secondary metabolite naphthoquinone, on the expression levels of *GRP75*, *TFAM* and *mTOR* genes related to mitochondrial activation and biogenesis in PANC-1 and BxPC-3 human pancreatic cancer cell lines were investigated. Our study results revealed that juglone has an opposite but strong effect especially on *mTOR* gene expression in both cell lines. This dual effect of juglone on both cell lines from different origins indicates that different molecular mechanisms and actors are effective in the development of cancer in two different cancer cell lines causing cellular response in very different directions to the treatment.

Our study is the first that investigated the effects of juglone on mitochondrial biogenesis and activation in pancreatic cancer cells and showed the significant effects of juglone on *mTOR* gene expression in both cell lines. The mitochondrion plays a central role with the regulation of metabolic plasticity and fission-fusion cycles on metastasis and resistance to chemotherapy and radiotherapy processes. Therefore, mitochondrial targeting seems to be important in the fight against pancreatic cancer and further studies are needed in this context and as previously reported, mTOR may be an important therapeutic target in pancreatic cancer. In addition, since juglone which is a promising agent in pancreatic cancer, has high toxic properties and low solubility; studies to increase its bioavailability by developing new application approaches will undoubtedly be extremely important.

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Author Contributions

Conception: H.A., Design: D.E.K., Data Collection and Processing: E.M.D.B., F.G., Supervision: H.A., D.E.K., Analysis and Interpretation: F.G., D.E.K., Literature Review: E.M.D.B., H.A., Writer: E.M.D.B., H.A., Critical Review: H.A.

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