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Matairesinol induced antiproliferative effects via mitochondrial dysfunction in HepG2 cells

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ABSTRACT: Hepatocellular carcinoma is one of the most severe and life-threatening types of cancer. The conventional treatment of hepatoma has significant challenges due to the adverse effects of chemotherapy, leading to treatment failure and decreased survival. Therefore, developing and investigating novel and safer anticancer drugs and establishing more effective therapeutic regimens is a crucial field of research. The *Haplophyllum megalanthum*-derived compound matairesinol has demonstrated antiproliferative and antitumor activity against various types of cancer, including pancreatic, breast, and prostate cancer. However, the potential effects of matairesinol on liver cancer, as well as the underlying molecular mechanisms associated with its anticancer activity, have yet to be thoroughly elucidated. In the current study, we demonstrated that matairesinol inhibited the viability of human hepatoma cells in a dose-dependent manner. Besides, at IC_{50} and higher doses, matairesinol induced oxidative stress and impaired mitochondrial membrane potential and ATP levels, two evidence of mitochondrial damage. Moreover, matairesinol exposure led to significant caspase-3 activation, a hallmark of apoptosis. These results indicate that mitochondrial damage and caspase-3 activation may contribute to the cytotoxic effect of matairesinol on liver cancer cells.

KEYWORDS: Matairesinol; liver cancer; mitochondria; caspase-3; anticancer effect.

1. INTRODUCTION

Hepatocellular carcinoma ranks fifth among cancer-related mortality worldwide, and it is assumed to remain one of the leading reasons of death in the near future. Hence, it is crucial to identify novel and effective molecular targets and develop new therapeutic approaches as soon as possible. The process of carcinogenesis is highly complex. Therefore, researchers are constantly investigating the effectiveness of different biomolecules in targeting cell death mechanisms or oncogenic pathways [1]. Natural compounds are mainly found in medicinal plants and are vital resources for uncovering new therapeutic agents. Several extensively used anticancer drugs are derived from natural sources, including plants (irinotecan, paclitaxel), marine sources (bleomycin) and bacteria (mitomycin C) [2]. Nowadays, combinations of different chemotherapeutic pharmaceuticals are preferred in different cancer types in order to enhance their effectiveness and overcome resistance mechanisms. Moreover, determining medication schedules presents a valuable possibility for minimizing potential toxicity concerns. It has also been suggested that the combination therapy alternative of adding natural products to schedules promotes an effective pharmacologic response and leads to fewer side effects [3]. Therefore, developing safer and more effective treatments for cancer also requires a thorough investigation of newly discovered natural compounds and a clear understanding of their molecular mechanisms that enable them to combat cancer.

Lignans are naturally occurring polyphenolic compounds in plants, including fruits, cereals, and seeds. Matairesinol is one of the essential dibenzylbutyrolactone-based lignans isolated from the fruit of Forsythia suspensa and the marine seagrass *Halophila stipulacea* [4]. The anti-inflammatory, antioxidant, and neuroprotective activities of matairesinol have been noted in various reports; however, anticancer properties

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need to be extensively investigated. In recent years, matairesinol has been emphasized as a promising anticancer agent in a range of cancer types, including prostate, pancreatic, and breast cancer [5-7]. Unfortunately, almost little information is known on the mechanisms underlying the antiproliferative effects of matairesinol in liver cancer cells. Therefore, the current study aimed to investigate the related biochemical and cellular mechanisms of matairesinol-induced cytotoxicity on HepG2 cells.

Therapeutic approaches targeting apoptotic mechanisms and mitochondrial structure/functions present a proper strategy to promote the death of cancer cells since dysregulation of apoptosis is thought to be a crucial hallmark of carcinogenesis. Mitochondrial dysfunction plays an essential role in intrinsic apoptosis [8]. Drugs or drug candidates that trigger intrinsic apoptosis increase oxidative stress and intra-mitochondrial calcium levels, significantly reduce mitochondrial membrane potential, and impair ATP production. This results in the release of cytochrome-c into the cytoplasm and activation of the caspase cascade in the cell. The activation of effector caspases leads to programmed cell death. Inducing mitochondrial dysfunction and apoptosis is a crucial therapeutic strategy to inhibit the survival and proliferation of cancer cells [9]. In the current study, we examined the molecular mechanisms contributing to the anti-proliferative effects of matairesinol in HepG2. Firstly, we identified the cytotoxic effects of matairesinol and determined the IC_{50} value. Cytotoxicity assay was also performed on healthy cells to reveal the selectivity of the compound against cancer cells. NIH/3T3, a fibroblast cell line, was used for this purpose. Then, we measured the effects of matairesinol on oxidative stress, mitochondrial membrane potential (MMP), ATP production and caspase-3 activation in HepG2 cells. The findings of this study may provide preliminary information for further mechanistic investigations on the anticancer effects of matairesinol in liver cancer.

2. RESULTS AND DISCUSSION

Mitochondria play an indispensable role in cancer therapy due to their involvement in redox homeostasis, ATP metabolism, and programmed cell death. Cytotoxic drugs that interfere with mitochondrial structure or function are widely employed in targeted anticancer strategies [10]. Natural agents targeting the mitochondrial pathway have received significant attention in recent years. Luteolin [11], ganoleuconin [12], isoquercitrin [13], and berberine [14] were demonstrated to increase mitochondrial oxidative stress and cause the loss of MMP in various cancer cells. Furthermore, the molecular mechanisms contributing to the antitumor effects of new and other compounds should also be comprehensively investigated to design more effective anticancer drugs and identify all relevant anticancer pathways. In our study, we investigated the potential anticancer activities of matairesinol and the molecular mechanisms underlying these activities in HepG2 cells. Chemical characterization and LC/MS and NMR spectrums of matairesinol were presented in the supplementary material (Figure S1-6).

2.1. Cytotoxic effects of matairesinol

First, we aimed to determine the IC_{50} concentrations of matairesinol on HepG2 and NIH/3T3 cells to determine the cytotoxic potential and selectivity of the compound. As seen in Figure 1, matairesinol remarkably decreased the cell viability of HepG2 in a dose-dependent manner. Although there is a dose-dependent relationship in NIH/3T3 cells, the observed cytotoxicity is comparatively moderate.

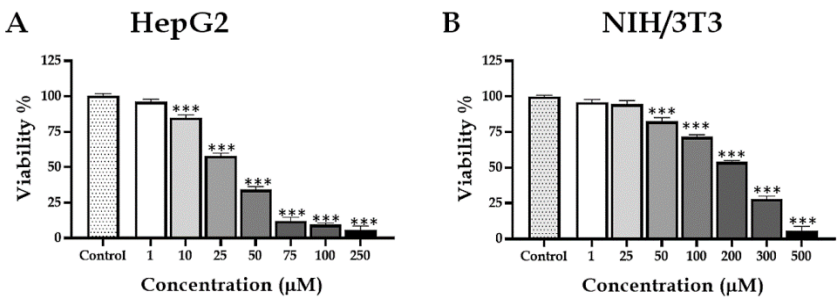

Figure 1. Cytotoxic effects of matairesinol assessed by MTT assay. A, HepG2 cells; B, NIH/3T3 cells. Each value is expressed as mean ± S.D. Viability was presented as a percentage of the solvent control (0.1% DMSO). *** $p<0.0001$, vs. control.

Remarkably, the calculated IC_{50} value of matairesinol in HepG2 cells was determined as 30.8 µM (Table 1). In addition to IC_{50}, we also calculated IC_{10} and IC_{75}, representing slightly and severely cytotoxic concentrations, respectively. The dose-response curve revealed that IC_{10} and IC_{75} values of matairesinol were 5.6 µM and 57.8 µM in HepG2 cells. Moreover, matairesinol weakly inhibited the viability of NIH/3T3 with an IC_{50} value of 227.1 µM, indicating that this compound exhibited a selective cytotoxic effect on HepG2

cancer cells (selectivity index: 7.4) without affecting healthy cells (Table). Further studies regarding the molecular mechanisms of matairesinol-induced cytotoxic effects were performed only on HepG2 cells.

Table 1. IC_{10}, IC_{50}, IC_{75} doses and selectivity index of matairesinol. The concentration values were determined from dose-response curves via GraphPad Prism® 8.4.2 software. The selectivity index is the ratio of the IC_{50} of NIH/3T3 to the IC_{50} of HepG2 cells.

Compound	HepG2			NIH/3T3	Selectivity index
	IC_{10} (µM)	IC_{50} (µM)	IC_{75} (µM)	IC_{50} (µM)	
Matairesinol	5.6	30.8	57.8	227.1	7.4

2.2. Oxidative stress and mitochondrial toxicity

After determining working concentrations, we aimed to investigate the underlying molecular mechanisms of antiproliferative effects of matairesinol on HepG2 cells. There have been limited studies and knowledge concerning the anticancer potential of matairesinol and molecular mechanisms of related antiproliferative effects. Lee et al. (2022) demonstrated that matairesinol induces mitochondrial depolarization, alters the related molecular mechanisms and induces apoptotic cell death in pancreatic cancer cells [15]. In another study, Peuhu et al. (2010) aimed to identify the involvement of the AKT pathway in the matairesinol-induced antitumor effects on prostate cancer cells [7]. However, the current knowledge regarding the anticancer activities and intracellular pathways involved in the potential effects of matairesinol on HepG2 cells remains unknown. In the present study, HepG2 cells were incubated with matairesinol at increasing dose levels to determine the contribution of oxidative stress, loss of MMP and ATP production to the observed cytotoxicity.

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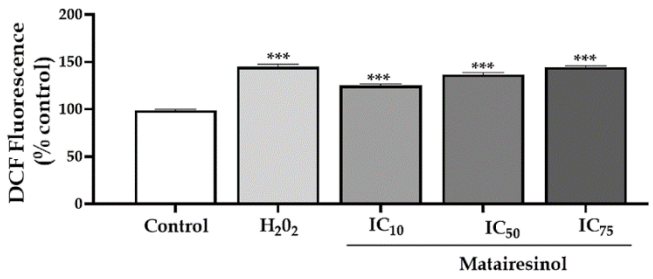

Figure 2. Matairesinol induced oxidative stress on HepG2 cells in a dose-dependent manner. Cells treated with increasing concentrations of compound (IC10, IC50, IC75) and oxidative stress were measured dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. All data was expressed percentage of control (DMSO, %0.1). H_20_2 (10µM) treated cells were accepted as a positive control. *** $p<0.0001$, vs. control.

As illustrated in Figure 2, matairesinol significantly increased the oxidative stress in HepG2 cells at IC_{10} and higher doses (Figure 2). The redox potential of cells plays a critical role in the progression of tumors via multiple tumorigenesis signaling pathways and profoundly influences the efficacy of cancer treatment. Reactive oxygen species (ROS)-mediated signaling is suggested to involve different aspects of cancer cell manners, including survival, proliferation, apoptosis, and metabolic adaptation, and therefore, oxidative stress is a crucial target for anticancer therapy [16].

The cellular state of redox is remarkable in initiating apoptosis [17]. In the intrinsic apoptotic pathway, various cellular signals trigger the increase in expression and activation of Bax, a pro-apoptotic protein belonging to the Bcl-2 family. Bax undergoes conformational modifications, binds to the mitochondrial outer membrane and oligomerizes. Excessive oxidative stress and Bax translocation lead to the disruption of the mitochondrial membrane, resulting in the release of cytochrome c from the mitochondria into the cytosol, formation of, activation of caspase-3, -9 and eventually, cell death [18]. Evaluating the potential contribution of excessive oxidative stress to mitochondrial dysfunction, we further examined the effects of matairesinol on MMP by 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine, iodide (JC-1) assay and ATP levels via the bioluminescence method. In HepG2 cells, treatment with matairesinol at a concentration of IC_{10}, which refers to the no-cytotoxic concentration level, did not significantly affect the mitochondrial membranes and ATP production (Figure 3A, B). In contrast, matairesinol exposure resulted in the loss of MMP and ATP levels by 28% and 37% at the IC_{50} dose Moreover, the higher dose (at IC_{75}) led to a dramatic increase in the impairment of mitochondrial membranes and ATP production; MMP and ATP levels were reduced by approximately 66% and 48%, respectively (Figure 3A, B). Mitochondrial damage can lead to irreversible and fatal cell damage in cancer cells, making our results crucial for understanding the anticancer effect mechanism of matairesinol.

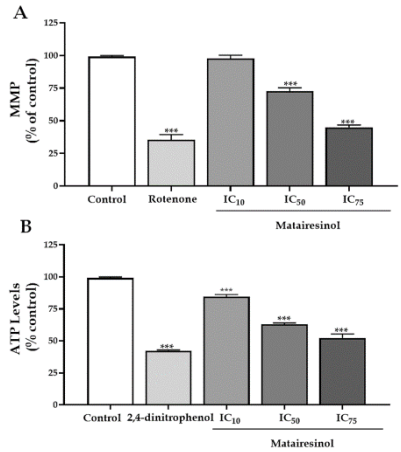

Figure 3. Matairesinol treatment led to mitochondrial dysfunction in HepG2 cells. A, Alterations in mitochondrial membrane potential (MMP) induced by matairesinol; B, The effect of matairesinol on ATP levels. All matairesinol incubations were conducted at again three dose levels. Rotenone and 2,4-dinitrophenol were used as positive controls for MMP and ATP analysis, respectively. The value of control (DMSO, %0.1) was accepted 100%, and all values were normalized to control. *** $p<0.0001$, vs. control.

2.3. Caspase-3 activity

Apoptosis is a highly regulated process, and one of its central events is the activation of caspase-3. This endoprotease is activated by caspase-8, which cleaves pro-caspase-3 and triggers the cleavage of multiple intracellular proteins. Caspase-3 is essential for apoptotic chromatin condensation and DNA fragmentation in cells and contributes to characteristic features of apoptosis [19]. Hence, the induction of caspase-3 activation has been identified as a promising approach for anticancer strategy. Additionally, the demonstration of caspase-3 activation is an essential hallmark for identifying and studying apoptosis. In our study, matairesinol at IC_{10} had no significant effect on the cleavage of pro-caspase-3 (Figure 4). In contrast, matairesinol led to considerable activation of caspase-3 at higher doses. Treatment with IC_{50} and IC_{75} concentrations of matairesinol resulted in a 4.2- and 5.1-fold increase in the related protease enzyme activity, respectively, indicating its substantial apoptosis inducer property (Figure 4).

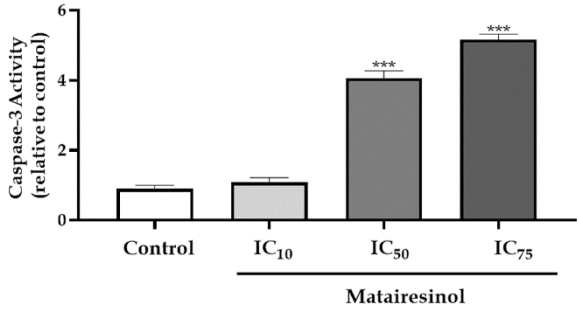

Figure 4. Matairesinol induced caspase-3 activation in HepG2 cells. Cells treated with DMSO (0.1%) were accepted control, and all results were normalized to control. *** $p<0.0001$, vs. control.

4. CONCLUSION

Our preliminary results indicated that mitochondrial damage and caspase-3 activation are involved in matairesinol-induced cytotoxic effects on HepG2 cells. Although a remarkable decrease in ATP levels, loss of MMP and activation of effector caspase are indicators of intrinsic apoptosis, further investigation is required to determine the precise mechanisms by which matairesinol mediates its anticancer activity and to evaluate its potential as a therapeutic target for liver cancer.

5. MATERIALS AND METHODS

5.1. Isolation and characterization of matairesinol

Matairesinol was isolated from the endemic plant *Haplophyllum megalanthum Bornm*. The aerial parts of *H. megalanthum* were collected from Manisa/Turkey and deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University. Air-dried and powdered plant material (15.9 kg) was

extracted with 96% ethanol. Crude ethanolic extract was dissolved in 2.5% hydrochloric acid. Then, the acidic filtrate was basified with ammonia to pH 9-10. The alkaline filtrate was extracted with chloroform to obtain the crude extract. The crude extract was fractionated by column chromatography employing silica gel 60. After applying the TLC controls, a total of 32 fractions were obtained [20]. Matairesinol was purified from fraction-8 by using reversed-phase Phenomenex Luna C18(2) column (150 x 4.6 mm i.d, 3 µm particle size, 100 Å pore size) and an analytical-scale Agilent 1100 series HPLC instruments consisting of a G1313A high-performance autosampler, a G1311A quaternary pump, a G1379A degasser, a G1316A thermostatted column compartment, a G1315B PDA detector and all controlled by Agilent ChemStation version B.03.02 software (Demir, S). The structure of matairesinol was elucidated by NMR and LC-MS experiments. The analyses were conducted using the conditions that were described in detail by Petersen et al. [21].

5.2. Cell culture and materials

HepG2 (human hepatoma) and NIH/3T3 (mouse embryonic fibroblast) cells were purchased from ATTC, cultured in DMEM-F12 supplemented with 10% FBS and kept in a humidified 5% CO2 incubator at 37 °C. Matairesinol, JC-1, DCFH-DA and other chemicals required for cell culture analysis were provided by Sigma-Aldrich (Darmstadt, Germany). The caspase-3 and ATP assay kits were applied from Thermo Fisher Scientific (E13183), and Promega Corp (G9241), respectively. Matairesinol dissolved in DMSO, and the final DMSO concentration was set at %0.1 in all experiments. Therefore, cells not treated with matairesinol but exposed to media containing only 0.1% DMSO were accepted as a control.

5.3. Cell viability assay

MTT assay was performed to evaluate the cytotoxic effects of MAT and determine the working concentration in our further analysis. HepG2 and NIH/3T3 cells (6×10^3 cells/per well) were incubated with matairesinol at increasing concentrations. After a 24-hour incubation period, 10µL of MTT dye was added to each well, mixed, and incubated for 4 hours at 37°C. The microplate reader was employed to measure the absorbance at 450nm [22]. IC_{10}, IC_{50} and IC_{75} values were calculated via dose-%viability graphs. The ratio of IC_{50}s of NIH3T3 and HepG2 cells defined the selectivity index of matairesinol.

5.4. Oxidative stress

The level of ROS within the cells was analyzed by utilizing the fluorescence probe DCFH-DA, which is sensitive to oxidation. Cells were seeded into a 96-well plate at a density of 6 × 103 cells/well and treated with three doses (IC_{10}, IC_{50}, IC_{75}) of matairesinol for 24 hours. H_2O_2 (10 µM) was applied as a positive control. During each incubation, DCFH-DA was added at a final concentration of 20 µM in the dark and incubated with cells for 30 minutes. Cells were then collected, washed in PBS and resuspended in 500 µL of PBS for fluorimetric analysis [23]. The ROS levels were measured using a microplate fluorescence spectrophotometer (Ex/Em: 485/530 nm).

5.5. Mitochondrial membrane potential

The potential of mitochondria's outer membrane is essential for the maintenance of mitochondrial functions. We utilized a fluorescent probe called JC-1 to assess the potential effects of matairesinol on MMP. The JC-1 probe is a cationic dye that accumulates in the mitochondrial matrix, where it undergoes a concentration-dependent shift in fluorescence from green to red due to its ability to form J-aggregates in response to the membrane potential. This shift in fluorescence can be measured to determine changes in MMP, which is a critical indicator of mitochondrial functions. In brief, after the exposure to matairesinol at different concentrations (IC_{10}, IC_{50} and IC_{75}) or rotenone (positive control; 25 µM), HepG2 cells were incubated with a JC-1 working solution (5 µg/ml) for 30 minutes. Subsequently, cells were washed with a related buffer solution. The fluorescence ratio of red to green was used to determine the alterations in MMP. The measurement was conducted at ex: 490 nm and em: 520 nm [24].

5.6. ATP levels

ATP level is another critical parameter of mitochondria functions. HepG2 cells were exposed to matairesinol (IC_{10}, IC_{50} and IC_{75} doses, 24 hours), and after the incubation period, cellular ATP levels were measured by assay kit. Cells treated with DMSO (0.1%) and 2,4-dinitrophenol (20µM) were used as a negative and positive control, respectively. After incubation, 40 µl of ATP assay reagent was added to each well. The plates were then shaken for 5 minutes and incubated for 30 minutes. Subsequently, the

luminescence was quantified utilising a microplate reader. The signal of luminescence obtained from the cells treated with DMSO (0.1%) was considered 100% [25].

5.7. Caspase-3 assay

The caspase-3 activity induced by matairesinol was carried out in HepG2 cells using a commercial kit. The observations from this assay contribute to understanding the potential effects of matairesinol on the induction of apoptosis in HepG2 cells. $6x10^3$ cells/well were incubated with IC_{10}, IC_{50}, or IC_{75} doses of matairesinol. The wells were treated with the reaction mix (50 µM) for 24 hours. Then, both control and sample wells were administered with 25µM Asp-Glu-Val-Asp-pNA (DEVD-pNA). The absorbance measurement was carried out using a multi-plate reader at a wavelength of 405nm. The caspase-3 activity in each well was revealed as a fold change compared to the control group [26].

5.8. Statistical analysis

The statistical analysis for the experimental data was conducted using GraphPad Prism 8.0. The "mean ± standard deviation (SD)" was calculated based on a minimum of three independent experiments. To compare between groups, one-way ANOVA was used for variance analysis. The standards for statistical significance were set at a p-value of less than 0.05.

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