Magnoflorine promotes Huh-7 cell apoptosis and autophagy by regulating PI3K/Akt/mTOR pathway

Jifan Xu¹, Bo Du¹, Yunfeng Liu¹, Chonglin Tao²*

¹Department of Hepatobiliary Surgery, Peoples Hospital of Chongqing Kaizhou, Chongqing, China; ²Department of Hepatopancreatobiliary Surgery, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China

*Corresponding Author: Chonglin Tao, Department of Hepatopancreatobiliary Surgery, The First Affiliated Hospital of Wenzhou Medical University, Shangcai Village, Baixiang Street, Ou hai District, Wenzhou, Zhejiang 325000, China. Email: cltao8185@163.com

Received: 23 November 2021; Accepted: 14 December 2021; Published: 20 January 2022

© 2022 Codon Publications

OPEN ACCESS

ORIGINAL RESEARCH

Abstract

Hepatoma is a malignant tumor with high rates of heterogeneity, metastasis, and mortality. Currently, there is no effective treatment available for hepatoma. In order to treat advanced hepatoma in a better manner, new and more effective therapeutic targets still need to be developed. Magnoflorine (MGN) is a quaternary ammonium alkaloid with a variety of therapeutic properties. MGN inhibited the proliferation of lung cancer, breast cancer, glioma, and rhabdomyosarcoma cells, induced apoptosis, and blocked cell cycle. However, its possible effects on the progression of hepatoma are still indefinite. In this study, the effects of MGN on the progression of hepatoma in vitro and the underlying mechanisms were determined. MGN suppressed the proliferation, induced the autophagy, and stimulated the apoptosis of human hepatoma Huh-7 cells. Mechanically, MGN could regulate PI3K/AKT/mTOR pathway, which therefore affects the progression of hepatoma in vitro. Taken together, MGN affected Huh-7 cell proliferation, autophagy, and apoptosis, and might act as a promising therapeutic drug for treating hepatoma.

Keywords: apoptosis; autophagy; hepatoma; Huh-7 cells; magnoflorine (MGN); proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies of the digestive tract (Hoshimoto et al., 2018; Luan et al., 2021). It ranks as the fifth most common malignancy after lung cancer and is the second most deadly tumor (Gailhouste et al., 2013; Wu et al., 2020a). The pathogenesis of HCC is a complex process involving multiple steps and factors, including environment, genetics, and lifestyle (Bao et al., 2018). Various risk factors and toxicants, including aflatoxins, nitrosamines, and trace elements, are closely associated with the development and progression of HCC (van Rensburg et al., 1990). The incidence of HCC is high, accounting for about 55% of patients worldwide (Jagric and Horvat, 2020). The mechanisms underlying the pathogenesis, progression, diagnosis, and treatment of HCC are still a major challenge in biomedical research (Li et al., 2021). Given the lack of obvious symptoms in early stages of HCC, patients are often already at an advanced stage by the time they seek medical attention (Shun et al., 2008). Yet, traditional treatment methods, such as surgical resection, radiotherapy, and chemotherapy, have limited effects (Shun et al., 2008). In order to better treat advanced HCC, there is still a requirement to develop new and more effective therapeutic targets.

Magnoflorine (MGN), also named as thalictrine, occurs naturally in the root of buttercup plant, and can be produced by chemical synthesis as well. It has neuropsychopharmacological, antianxiety, immunomodulatory, anti-inflammatory, antioxidant, and antifungal activities (Chang et al., 2020; Okon et al., 2020a, 2020b). MGN ameliorates inflammation and fibrosis in rats.
with diabetic nephropathy via regulating the stability of LSD3A (Chang et al., 2020). MGN inhibited the proliferation of breast cancer, gastric cancer, and rhabdomyosarcoma cells in a dose-dependent manner as well as induced cell apoptosis and blocked cell cycle (Okon et al., 2020a, 2020b; Sun et al., 2020c). Studies have shown that magnolina induces cell autophagy, cell apoptosis, and cell cycle arrest to inhibit the development of gastric cancer (Wang et al., 2020). MGN can also induce apoptosis and autophagy of breast cancer cells through Akt/mTOR signaling pathway (Wei et al., 2020). However, its possible effects on the progression of HCC are still indefinite.

The PI3K/Akt/mTOR pathway is closely associated with cell proliferation, apoptosis, autophagy, and inflammation (Sun et al., 2020b). mTOR is an important downstream regulator in the PI3K/Akt pathway, and plays a crucial role in protein synthesis and autophagy (Golob-Schwarzl et al., 2017; Seshadri, 2021). Increasing evidences have suggested that several factors can inhibit progression of HCC through inactivation of PI3K/Akt/mTOR signaling pathway (Golob-Schwarzl et al., 2017). In this study, the effects of MGN on the progression of Huh-7 cell proliferation, autophagy, and apoptosis were determined in vitro.

Materials and Methods

Antibodies and drugs

Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:3,000, ab8245; Abcam, Cambridge, MA, USA), LC3B antibody (1:1,000 dilution, #4108; Cell Signaling Technology (CST) Inc., Beverly, MA, USA), Beclin-1 antibody (1:500 dilution, #3495; CST), P62 antibody (1:1,000 dilution, ab207305; Abcam), p-PI3K (1:1,000, ab278545; Abcam), PI3K (1:1,000, ab154598; Abcam), p-AKT (1:1,000, ab38449; Abcam), AKT (1:1,000, ab8505; Abcam), p-mTOR (1:500, ab137133; Abcam), mTOR (1:1,000, ab134903; Abcam), Caspase 3 (1:1,000, ab32351; Abcam), Caspase 9 (1:1,000, ab32359; Abcam), cleaved caspase 3 (1:1,000, ab32042; Abcam), cleaved caspase 9 (1:1,000, ab2324; Abcam), and Bcl-xl (1:500, ab137133; Abcam). Magnoflorine (CAS 2141-09-5) was purchased from ChemGenes Corp (MA, USA) and dissolved in dimethyl sulfoxide (DMSO). The final concentrations of MGN in Huh-7 cell line were 0, 20, 40, and 80 µM.

Cell culture and transfection

The human normal hepatoma cell line, Huh-7, was purchased from ATCC and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO₂ incubator. MGN was administrated into Huh-7 cell line at the concentrations of 0, 20, 40, and 80 µM for 24 h.

Cell counting kit-8 (CCK-8) assay

Huh-7 cells were plated in 96-well plates with 1,000 cell/well density and maintained for 72 h upon the indicated treatment. Cells were then incubated with CCK-8 for 4 h and the OD value was measured at 0, 24, 38, and 72 h at 490-nm wave length.

Immunoblot assay

The immunoblot assay was performed according to the study conducted by Wei et al. (2020). Huh-7 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer. Cells in different groups were isolated and the proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% fat-free milk. Membranes were incubated with both primary antibodies for 1.5 h and secondary antibodies for another 1 h. Signals were detected using an enhanced chemiluminescent (ECL) kit.

Enzyme-linked-immunosorbent serologic assay (ELISA)

ELISA was performed according to the study conducted by Wei et al. (2020). The effects of MGN on the activities of caspase-3 (ab252897; Abcam) and caspase-9 (ab219915; Abcam) in Huh-7 cells were detected according to the imparted instructions.

Cell cycle and apoptosis assays

For apoptosis assay, hepatoma cells were fixed with 70% ethyl alcohol for 24 h at -20°C and treated with Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 20 min, then analyzed using a flow cytometer. For cell cycle assay, Huh-7 cells were fixed with 70% ethyl alcohol for 24 h at -20°C and incubated with PI for 20 min. The percentage of cells was analyzed.

Statistics

GraphPad 6.0 was used for analysis. Data were shown as mean ± standard error of mean (SEM). One-way ANOVA was used for data comparison, and \( P < 0.05 \) was considered statistically significant. * indicates \( p < 0.05 \), ** \( p < 0.01 \), and *** \( p < 0.001 \), respectively.
Results

MGN suppressed the proliferation of Huh-7 cell line

In order to uncover the possible effects of MGN on the progression of hepatoma, its effects were determined at different concentrations of 0, 20, 40, and 80 μM on the proliferation of hepatoma cell line Huh-7. CCK-8 assays showed that MGN treatment suppressed the proliferation of Huh-7 cells in a concentration-dependent manner (Figure 1A). The flow cytometry (FCM) assay showed that treatment of MGN led to arrest of Huh-7 cell cycle, with the increased percentage of cells at G1 phase and decreased percentage of cells at S phase (Figures 1B and C). Therefore, MGN treatment inhibited the proliferation of Huh-7 cells through stimulating cell cycle arrest.

MGN treatment induced the autophagy in Huh-7 cell line

Further study was intended to detect the effects of MGN on the autophagy of hepatoma cells. The immunoblot assay showed that the expression of p62, a protein that can negatively regulate autophagy, was decreased after MGN treatment (Figure 2). Besides, the expression of beclin-1 was upregulated upon the treatment of MGN, suggesting the activation of autophagy (Figure 2). The ratio of LC3II:LC3I was also increased after MGN treatment, which further confirmed that MGN treatment induced autophagy in Huh-7 cells (Figure 2).

MGN treatment stimulated the apoptosis of Huh-7 cell line

The previous results detected the effects of MGN on the apoptosis of hepatoma cells by FCM assay. Interestingly, MGN treatment significantly stimulated the apoptosis of Huh-7 cells in a concentration-dependent manner (Figures 3A and B). ELISA suggested that both caspase-3 and caspase-9 were dramatically activated after MGN treatment in Huh-7 cells (Figure 3C). Immunoblot assay was performed to detect the protein expressions of apoptosis-related proteins upon MGN treatment at various concentrations (20, 40, and 80 μM). The results showed the up-regulation of cleaved caspase-3 and cleaved caspase-9, and the down-regulation of Bcl-xl upon MGN treatment, confirming the activation of caspase (Figure 3D). Therefore, treatment of MGN stimulated the apoptosis of Huh-7 cells.

MGN treatment inactivated PI3K/AKT/mTOR pathway in Huh-7 cell line

It is well accepted that cell proliferation, autophagy, and apoptosis could be mediated by PI3K/AKT pathway. Therefore, the involvement of PI3K/AKT pathway in the regulatory roles of MGN in hepatoma cell proliferation, autophagy, and apoptosis was validated. The immunoblot assay showed that MGN treatment suppressed the phosphorylated levels of PI3K and AKT in Huh-7 cells (Figure 4).

Figure 1. Magnoflorine suppressed the proliferation of Huh-7 cell line. (A) CCK-8 assay demonstrated the effects of MGN treatment on the proliferation of Huh-7 cells at 0-, 20-, 40-, and 80-μM concentrations, and the OD value at 490-nm wavelength was quantified. (B) FCM assay demonstrated the effects of MGN treatment on Huh-7 cell cycle at 0-, 20-, 40-, and 80-μM concentrations. (C) The quantification of (B). Data were presented as mean ± SEM; *P < 0.05, **P < 0.01.
Figure 2. Magnoflorine treatment induced the autophagy of Huh-7 cell line. Immunoblot assay demonstrated the expression of proteins on Huh-7 cells after MGN treatment at 0-, 20-, 40-, and 80-µM concentrations. Data were presented as mean ± SEM; *P < 0.05, **P < 0.01.

Figure 3. Magnoflorine treatment stimulated the apoptosis of Huh-7 cell line. (A) FCM assay demonstrated the effects of MGN treatment on the apoptosis of Huh-7 cells at 0-, 20-, 40-, and 80-µM concentration. (B) The quantification of (A). (C) ELISA demonstrated the activities of Caspase-3 and Caspase-9 in Huh-7 cells on MGN treatment at 0-, 20-, 40-, and 80-µM concentrations. (D) Immunoblot assay demonstrated the expression levels of proteins on Huh-7 cells after MGN treatment at 0-, 20-, 40-, and 80-µM concentrations. Data were presented as mean ± SEM; *P < 0.05, **P < 0.01.
Magnoflorine suppresses hepatoma development

Antioxidant activities (Chang et al., 2020; Liang et al., 2020; Okon et al., 2020b). For example, MGN had the potential to treat 2,4-Dinitrochlorobenzene (DNCB)-induced atopic dermatitis by suppressing the apoptosis of keratinocyte (Wu et al., 2020b). MGN also suppressed mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathway to suppress inflammatory osteolysis induced by titanium particles (Sun, et al., 2020a). Importantly, MGN suppressed the progression of multiple types of cancers (Sun et al., 2020c; Wei et al., 2020). MGN also inhibited progression of gastric cancer by inducing autophagy, apoptosis, and cell cycle arrest by c-Jun N-terminal kinase (JNK) activation regulated by reactive oxygen species (ROS) (Seshadri, 2021). These studies, together with our findings, confirmed that MGN could be an effective drug for treating hepatoma.

It is also noticed the PI3K/AKT/mTOR pathway mediated the proliferation, apoptosis, and autophagy of hepatoma cells. In fact, PI3K/AKT/mTOR pathway is known to be related with the progression of various cancers (Golob-Schwarzl et al., 2017; Guo et al., 2021). For example, YTHDF1 could promote HCC via activating PI3K/AKT/mTOR pathway and inducing epithelium to mesenchymal transition (Luo et al., 2021). Brucine promoted the apoptosis and suppressed the proliferation of cervical cancer cells by PI3K/AKT/mTOR pathway (Seshadri, 2021). Long non-coding RNAs (LncRNAs), such as microRNA-19b, could affect oxaliplatin chemoresistance in colon cancer through PI3K/AKT/mTOR pathway (Fan et al., 2021). These studies, together with our findings, confirmed that MGN could be an effective drug for treating hepatoma.

Discussion

In the present study, effects of MGN were revealed on the progression of Huh-7 cell proliferation, autophagy, and apoptosis. The results established that MGN could serve as a promising drug for HCC treatment. HCC is a malignant tumor with a high rate of morbidity, metastasis, and recurrence (van Rensburg et al. 1990). Although its etiology and pathogenesis have not been well explored, with early diagnosis and treatment of primary hepatoma, the overall outcomes have improved significantly (Hoshimoto et al., 2018). However, in advanced HCC, surgical resection, radiotherapy, and chemotherapy are not effective in achieving a satisfactory outcome (Galicia-Moreno et al., 2021). Even after radical resection of HCC, ~60–70% patients still develop metastasis and recurrence within 5 years (Bao et al., 2018; Nurili et al., 2021). Urgent requirement is sought to find new and more effective drugs for the treatment of HCC. Here, a quaternary ammonium alkaloid, MGN, was considered a potential factor, based on its effects on the proliferation, autophagy, and apoptosis of hepatoma cells in vitro.

CCK-8 and FCM assays demonstrated that MGN suppressed the proliferation of hepatoma cells by stimulating cell cycle arrest. Immunoblot assay validated that MGN could affect the autophagy of hepatoma cells. FCM assay and ELISA suggested that MGN induced hepatoma cell apoptosis. These findings confirmed the antitumor role of MGN in hepatoma. MGN had multiple biological traits, such as antitumor, anti-inflammatory, and antioxidant activities (Chang et al., 2020; Liang et al., 2020; Okon et al., 2020b). For example, MGN had the potential to treat 2,4-Dinitrochlorobenzene (DNCB)-induced atopic dermatitis by suppressing the apoptosis of keratinocyte (Wu et al., 2020b). MGN also suppressed mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathway to suppress inflammatory osteolysis induced by titanium particles (Sun, et al., 2020a). Importantly, MGN suppressed the progression of multiple types of cancers (Sun et al., 2020c; Wei et al., 2020). MGN also inhibited progression of gastric cancer by inducing autophagy, apoptosis, and cell cycle arrest by c-Jun N-terminal kinase (JNK) activation regulated by reactive oxygen species (ROS) (Seshadri, 2021). These studies, together with our findings, confirmed that MGN could be an effective drug for treating hepatoma.

In addition, the phosphorylated level of mTOR was also decreased after MGN treatment in Huh-7 cells (Figure 4). Therefore, MGN treatment inactivated PI3K/AKT/mTOR pathway in Huh-7 cells.

![Figure 4. Magnoflorine treatment inactivated the PI3K/AKT/mTOR pathway in Huh-7 cell line. Immunoblot assay demonstrated the expression levels of indicated proteins in Huh-7 cells after MGN treatment at 0-, 20-, 40-, and 80-µM concentrations. Data were presented as mean ± SEM; **P < 0.01.](image-url)
motility through PI3K/AKT/mTOR signaling pathway, which needs further validation. A previous study has revealed that MGN improved the sensitivity of breast cancer cells to doxorubicin (DOX) by inducing apoptosis and autophagy through AKT/mTOR pathway (Wei et al., 2020). Consistently, the regulatory role of MGN in AKT/mTOR pathway was observed in Huh-7 cells.

Conclusion

The effects of MGN on the progression of hepatoma in vitro were established through by suppressing proliferation, inducing autophagy, and stimulating apoptosis in HCC cells. Mechanically, MGN could regulate the PI3K/AKT/mTOR pathway, and therefore affect Huh-7 cells in vitro. MGN could thus act as a promising therapeutic drug for treating hepatoma treatment.

Competing Interests

The authors state that there was no conflict of interest to disclose.

Author Contributions

Jifan Xu and Bo Du designed the study and supervised data collection. Yunfeng Liu analyzed and interpreted the data. Chonglin Tao prepared and reviewed the draft of manuscript for publication. All authors read and approved the final manuscript.

References


Magnoflorine suppresses hepatoma development


