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Protective effects of *Celastrol* on diethylnitrosamine-induced hepatocellular carcinoma in rats and its mechanisms

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ABSTRACT

Celastrol, an active ingredient of *Tripterygium Wilfordii*, is a traditional Chinese medicinal herb, which has attracted interests for its potential anti-inflammatory and anti-cancer activities. The aim of this study was to evaluate the anti-tumor effect of *Celastrol* against diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) in rats and furthermore, to explore the underlying mechanism. Sprague-Dawley rats were intragastrically administered with DEN (10 mg/kg) for 6 days every week and persisting 16 weeks. The number of nodules was calculated. Hematoxylin-Eosin (HE) staining was used to evaluate the hepatic pathological lesions. The levels of serum alanine aminotransferase (ALT), glutamic oxalacetic transaminase (AST), alkaline phosphatase (ALP) and alpha fetoprotein (AFP) were analyzed by Elisa kits, and the protein levels of p53, Murine double minute (MDM) 2, Bax, Bcl-2, Bcl-xl, cytochrome C, Caspase-3, Caspase-9 and Poly (ADP-ribose) polymerase (PARP) were analyzed by western blot. The results showed that *Celastrol* could significantly decrease the mortality, the number of tumor nodules and the index of liver in the *Celastrol* groups compared with DEN-treated group. Moreover, *Celastrol* obviously improved the hepatic pathological lesions and decreased the elevated levels of ALT, AST, ALP and AFP. Meanwhile, *Celastrol* suppressed the expression of the protein MDM2, activated the intrinsic mitochondrial apoptosis pathway induced by p53, inhibited anti-apoptotic Bcl-2 and Bcl-xl, induced the pro-apoptotic Bax, cytochrome C, PARP and caspases. These results suggested that *Celastrol* had a good therapeutic action in reversing DEN-induced HCC rats, which may be associated with the apoptosis of hepatoma cells induced by *Celastrol*.

1. Introduction

According to the International Agency for Research on Cancer, the GLOBOCAN 2012 shows that liver cancer is the sixth most common cancer in the worldwide. Thereinto, hepatocellular carcinoma (HCC) is the main common form of liver cancer with very poor prognosis and high mortality (El-Serag, 2012; Llovet et al., 2003). Due to the growing incidence, poor prognosis and hidden development of HCC (Zheng et al., 2014; Flores and Marrero, 2014), the effective therapy for HCC is still in challenging. Therefore, there is an urgent quest for searching for highly efficient anti-tumor drugs for HCC patients.

Thunder of God Vine (Tripterygium Wilfordii Hook F.) is a perennial vine of the Celastraceae family. The plant is poisonous but its root pulp contains several therapeutically active compounds, including steroids, alkaloids, and terpenoids. Celastrol and triptolide are the two main compounds extracted from Thunder of God Vine, which share several common properties but have different toxic effects (Salminen et al., 2010; Mou et al., 2011; Jiang et al., 2015). *Celastrol*, a plant triterpene which has much lower toxicity than triptolide and attracted great attention recently due to its significant anti-inflammatory and anti-cancer activities (Petronelli et al., 2009; Yang et al., 2014). The molecular formula of *Celastrol* is $C_{29}H_{38}O_4$ and its chemical structure is provided in Fig. 1A. Recent studies have demonstrated that *Celastrol* could inhibit tumor cell proliferation and induce apoptosis *in vitro* including breast cancer cells, gastric cancer cells, non-small-cell lung cancer cells (Jang et al., 2011; Mou et al., 2011; Lee et al., 2014). Our

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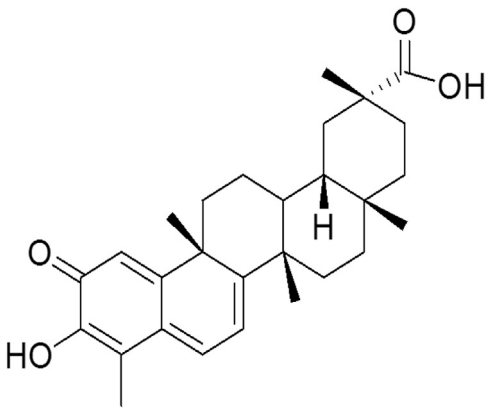

Fig. 1. The chemical structure of *Celastrol*.

previous studies had also found that *Celastrol* could induce Bel-7402 cells apoptosis by mitochondrial apoptosis pathway (Li et al., 2015). Furthermore, *Celastrol* has been found to suppress tumor initiation, progression in various cancer models *in vivo* (Yang et al., 2006; Huang et al., 2008; Jiang et al., 2013). However, the anti-cancer effect of *Celastrol* on DEN-induced HCC in vivo and its molecular mechanisms have never been conclusively shown.

Apoptosis is a strictly controlled mechanism of cell suicide and is critical for cytotoxicity induced by anti-cancer drugs (Cotter et al., 2009). The tumor suppressor p53 is a powerful growth-suppressive and pro-apoptotic protein, which plays a role in cellular apoptosis protecting organisms from developing cancer (Katiyar et al., 2000; Purvis et al., 2012; Ou et al., 2015). Murine double minute (MDM) 2 protein is an important regulator of the p53 pathway, which plays a central role in cancer development and progression (Javid et al., 2015; Graves et al., 2012). MDM2 directly binds p53, blocking the p53 transactivation domain and promoting its degradation (Shmueli and Oren, 2007). Many studies have confirmed the important role of p53 and MDM2 in the development and progression of HCC (Yang et al., 2013; Meng et al., 2014). P53 participates directly in the intrinsic apoptosis pathway through interacting with the members of Bcl-2 family (Ha et al., 2016; Vaseva et al., 2009). Bax and Bcl-2 are the major members of Bcl-2 family whose potential roles in tumor progression and prognosis of different human malignancies have been studied during the last decade. Bax promotes cell death through increasing the permeabilization of mitochondrial outer membrane, inducing the release of cytochrome C into the cytoplasm and then activating the caspases to promote the cleavage of several key cellular substrates. In contrast, Bcl-2 prevents apoptosis by inhibiting the activity of Bax (Mohan et al., 2012; Hector et al., 2009). Therefore, Bax/Bcl-2 ratio could respect the extent of apoptosis.

Diethylnitrosamine (DEN) is a well-known chemical agent that has a potent hepatocarcinogenic effect that is used to induce HCC in an experimental rat model. DEN-induced HCC has specificity on liver, high success rate and well repetitive rate (Yoshiji et al., 2002) and is similar to human HCC. Accordingly, we selected this model to investigate the anti-tumor effect of *Celastrol* on DEN-induced HCC rats and detected the levels of p53, MDM2, PARP, cytochrome C, Caspase-3, Caspase-9, Bcl-xl, Bax and Bcl-2 to explore the possible underlying molecular mechanisms.

2. Materials and methods

2.1. Drugs and reagents

Celastrol was purchased from Tiansheng Pharmaceutical Co. (Jurong, P. R. China). Diethylnitrosamine (DEN) was purchased from Sigma (St. Louis, MO, USA). p53, MDM2 and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PARP and cytochrome C were purchased from Abcam (Cambridgeshire, England, UK). Caspase-3, caspase-9 and Bcl-xl were purchased from Cell Signaling Technology (Colorado, USA). Bcl-2 and Bax were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, P. R. China). MMP-2 and MMP-9 were purchased from Biosynthesis Biotechnology (Beijing, P. R. China). The kits for ALT, AST, ALP and AFP were purchased from Beihua kangtai Biotechnology (Beijing, P. R. China). All other reagents were purchased in the purest from available.

2.2. Animals and treatment

Male Sprague-Dawley rats, weighing 140 ± 10 g, were obtained from the Laboratory Animal Centre of Anhui Medical University and maintained at an animal facility under pathogen-free conditions. All experiments were approved by Ethics Review Committee for Animal Experimentation of Institute of Clinical Pharmacology, Anhui Medical University. After acclimating for 7 days, rats were randomly divided into five groups, each containing fifteen rats as the following: (i) Normal control group. Rats were intragastrically administrated with saline. (ii) DEN-treated group. Rats were intragastrically administrated with DEN (10 mg/kg) for 6 days every week and persisting 16 weeks. (iii) *Celastrol*-treated groups. Rats were intragastrically administrated with the same DEN as DEN-treated group and administrated *Celastrol* (2, 4, 8 mg/kg) at the 10th week persisting 10 weeks. At 20th week, rats in each group were killed and liver specimens were removed and weighed. Serum samples were collected from the blood by centrifugation at 654*g* for 10 min at 4 °C for biochemical analysis. Macroscopically visible liver tumors and nodules greater than approximately 1 mm in diameter on liver surface were recorded. The right lobe of each liver was fixed in formalin solution for histopathological examination. The remaining liver tissues were stored at −80 °C for biochemical and western blot assays.

2.3. Histopathology analysis

Liver samples from the same liver lobes were fixed in 10% formalin immediately and embedded in paraffin. Then, the sections were stained with hematoxylin and eosin (H&E). The changes of liver pathological and the extent of liver tumors were observed and diagnosed under a microscope.

2.4. Measurement of serum AST, ALT, ALP and AFP levels

Serum was separated from the rat blood in a centrifuge under 654*g* for 10 min at 4 °C. The serum level of AFP, AST, ALT and ALP was measured using commercially available kits.

2.5. Calculation of liver index

Liver was removed and weighed. Liver index was calculated as liver weight divided by body weight (mg/g).

2.6. Western blot analysis

Liver fragments were lysed in RIPA (1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1 mM PMSF and complete protease inhibitor mix in PBS) buffer. Lysates were centrifuged at 14,000*g* for 15 min at 4 °C and the supernatants were diluted to 4 mg protein/mL and kept frozen at −80 °C. A total of 50 μg of denatured protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (PVDF membrane, Millipore, USA) in an ice-