European Journal of Pharmacology 796 (2017) 233–241

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Tanshinone IIA induced cell death via miR30b-p53-PTPN11/SHP2 signaling pathway in human hepatocellular carcinoma cells

Xuanqi Ren[a,1], Cui Wang[a,1], Binbin Xie[a], Linfeng Hu[a], Hui Chai[a], Lei Ding[a], Lihua Tang[a], Yongliang Xia[b,*], Xiaobing Dou[a,*]

[a] College of Life Science, Zhejiang Chinese Medical University, Hangzhou 310053, China
[b] Zhejiang Provincial Hospital of Traditional Chinese Medical, Hangzhou 310006, China

ARTICLE INFO

Keywords:
Tanshinone IIA
miR30b-p53
PTPN11
HepG2 cells
Cell death

ABSTRACT

Tanshinone IIA, a multi-pharmaceutical compound from traditional Chinese herb, has been reported to have anti-hepatocarcinomic (HCC) properties through cell death induction. Apart from the typical p53-dependent pathway, mechanisms of the anti-carcinogenic role of Tanshinone remain scarce. In an effort to explore the mechanism behind Tanshinone IIA, we detected the upstream of the p53 and the potential novel pathway. Tanshinone IIA dose-dependently initiated HepG2 cell apoptosis and cell cycle arrest at the G1 checkpoint. In the miR30 family, only the transcription of miR30b was downregulated by Tanshinone IIA, which subsequently upregulated both the genomic and protein levels of p53. Further, we screened that PTPN11 and Tp53 are the two critical genomes involved in the pharmacology of Tanshinone IIA. Building upon LASAGNA-search and kinetics binding assay, p53 was found to be a potential transcription factor for PTPN11. Concomitant with the expression of p53, Tanshinone IIA stimulated both PTPN11 and its encoded protein SHP2. Inhibition miR30b attenuated the Tanshinone IIA-induced cytotoxicity, level of p53 and PTPN11 in HepG2 cells. Finally, the apoptotic molecules such as Bax/Bcl2, cleavage caspase 3 and the cell cycle regulation factors including p21, cyclin D1, and CDK6 were changed by Tanshinone IIA. Several cytotoxic endpoints induced by Tanshinone IIA were also checked in Hep3B cells. This study confirmed that Tanshinone IIA may induce hepatoma cell death through the miR30b-p53- PTPN11/SHP2 pathway. With regard to the complicated tumorigenesis of HCC and the multi-targets of Tanshinone IIA, our results propose developing Tanshinone IIA for clinic therapy and the interference of HCC.

1. Introduction

Globally, the incidence rate of liver cancer has continued to increase over the past several decades. Worldwide liver cancer death rose from 695,900 to 745,500 from 2008 to 2012, with an estimated half of the deaths occurring in China (Jemal et al., 2011; Torre et al., 2015). Developing liver cancers, especially hepatocarcinoma (HCC), involved multiple biological signaling pathways (Whittaker et al., 2010). The complexity of the molecular pathogenesis of HCC poses great difficulties on finding a cure, and consequently discovering a multi-target medicine has been a long-lasting challenge for pharmacologists.

Traditional Chinese Medicine (TCM), well known for its multi-pharmacological intervention against a wide range of diseases (Hua et al., 2013), has endowed us the experience of therapy. Tanshinone IIA, the majority of extracts from the root of the Chinese herb *Salivia multiotthize*, served as a cardiprotective (Liu et al., 2013a, 2013b), a hepatoprotective (Wei et al., 2013), and even as an anti-carcinogen in clinic (Yu et al., 2014; Kapoor, 2009). Few literatures reported that Tanshinone IIA induced hepatic cancer cell death is primary through p53-dependent mitochondrial apoptotic pathway (Dai et al., 2012; Jeon et al., 2015). However, the upstream for Tanshinone IIA-related HCC cell death has yet to be uncovered and studies concerning on other molecules has been limited.

MicroRNA is a group of non-coding small molecules that regulate post-transcriptional gene expression. By controlling mRNA degradation and translation, miRNA plays a pivotal role in biological behavior (He et al., 2007; Ambros, 2004; Ebert and Sharp, 2012) To date, about half of miRNA are estimated to have been located in the cancer-associated genomic region (Calin et al., 2004). Furthermore, miRNA has been proven to be an oncogene and tumor suppressor in various types of cancers (Liao et al., 2014; Liu et al., 2016). Among hundreds of miRNA, miR30 has been referred as a core for miRNA oncogenetic

* Corresponding authors.
E-mail addresses: xiayongliang1@sina.com (Y. Xia), xbdou77@163.com (X. Dou).
[1] The authors made equal contributions to this work.

http://dx.doi.org/10.1016/j.ejphar.2016.11.046
Received 29 August 2016; Received in revised form 24 November 2016; Accepted 25 November 2016
Available online 26 November 2016

signaling pathways, especially in solid tumors (Volinia et al., 2010). Molecular p53 was the target of one conger of miR30 (Li et al., 2010). However, whether Tanshinone IIA regulated miR30 family is unknown.

Today, the modern development of bioinformatics offers a good opportunity to understand the holistic effects of medicine such as Tanshinone IIA. Bioinformatics is an emerging field that has helped scientists to computationally map the entire human genome. Information extracted and analyzed with biological tools can effectively indicate the underlying information hidden behind the available biological data which has helped to reveal the in-depth interaction (Gu and Chen, 2013). It has also been recognized as a convenient and time-saving technique to uncover the pharmaceutical information of TCM when compared with, though not replacing, and biological experiments.

Taken together, this study aims to elucidate the new pathway of Tanshinone IIA induced cancer cell death. We found that Tanshinone IIA unregulated p53 by miR30b inhibition. Then we found p53 is a specific transcription factor of PTPN 11 which acted as a core in the pharmacology of Tanshinone IIA. The signaling pathway of miR3b-p53-PTPN11/SHP2 pathway may be a new target for Tanshinone IIA in HepG2 cells.

2. Materials and methods

2.1. Materials

Tanshinone IIA (> 98.0% pure) was purchased from Sigma-Aldrich (USA). The antibodies, including p53, bcl2, and SHP2 were obtained from Abcam (Cambridge, UK); the mRNA sequence for Tp53, PTPN11, miR30a, b, c, and d, Bax, Bcl2, and p21 were devised by oligo primer sequence design 5.0. The Annexin V-FITC staining kits and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) solution was brought from CWbio (Beijing, China); The Cell culture medium DMEM and fetal bovine serum (FBS) was from hyclone.

2.2. Cell culture and treatment

Liver hepatocellular cells HepG2 and Hep3B originating from ATCC were obtained from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were maintained in DMEM containing 10% FBS at 37 °C, 5% CO_2. When confluent, cells were treated with a series of doses of Tanshinone IIA. After 24 h of exposure, the culture medium and cells were collected and prepared for the following bioassay. For the miR30b inhibition assay, cells were transfected with 50 nM of miR30b negative sequence and miR30b inhibitor sequences by Lipfectamine™ 2000 (Invitrogen) for 2 h before treated with Tanshinone IIA according to the protocol of the manufacturer (Riobobio, Guangzhou, China).

2.3. RT-PCR assays

Total RNA was extracted from cells by Trizol method (Invitrogen). The quality of RNA was determined by nanodrop (Thermo scientific, USA). Amplification of the corresponding genes was performed by Applied Biosystems (Foster City, CA). The data were calculated by $2^{-(\triangle\triangle CT)}$ and analyzed for fold induction of each gene as compared with the blank sample.

2.4. Western blot analyses

Total cell protein was isolated with RIPA lysate with 1 mM Mphenylmethanesulfonyl fluoride (PMSF). Protein concentration was determined by BCA kit as directed by the instructions. Approximately 50 μg of proteins were separated on SDS-10% polyacrylamide gels and then transferred to polyvinylidene diflouride (PVDF) membranes. After being blocked in 5% nonfat skim milk, the membranes were incubated with the antibody overnight at 4 °C. On the following day, the membrane was hybridized with a secondary antibody. Proteins were visualized by using Chemiluminescence Reagent and observed at Chemiscope (Clinx Science instruments Co. Ltd) and the software ChemiAnalysis was used to calculate the density of the protein bands.

2.5. Annexin V-FITC staining

Cells were collected and stained with 5 μl of Annexin V-FITC and 10 μl of PI. After incubation for at least 5 min in the dark, the apoptotic cells were analysis by flow cytometry.

2.6. Data discovery

Data mining was used to find the correlations among dozens of published literatures on the factors in which we are interested. The factors involved in our study were related to Tanshinone IIA. In total, there were 59 literatures and 61 genes after being filtered by species and repeated genes.

GeneCodis is a biological database which integrates various analytical tools and data. It has been widely applied to discover the function of genes that integrate different source of information in the same analysis, and has been used to discover the modular patterns. We used GeneCodis to perform the Go accumulation analysis and pathway annotation.

GeneMania imbedded with cytoscape can draft the interactions of the interested 61-listed genes through. The network of 61-selected genes was generated automatically by GeneMania and visualized by Cytoscape.

Gene2Networks integrated the content of ten mammalian interaction network datasets. Reconstruction of associated images was performed by Gene2Networks with Cytoscape. In order to further determine the affected pathways by Tanshinone IIA, certain pathways, which were identified to be associated with this research, were redrawn in Cytoscape in order to better compare these factors.

2.7. Transcription factors screening assay

LASAGNA-Search 2.0 which offers 1792 transcription factor models and 15 species for promoter retrieval is a web tool for TF binding site research available at http://biogrid-head.engr.uconn.edu/lasagna_search/ [LASAGNA-Search2.0: integrated transcription factor binding site search and visualization in a browser]. In this study, we used LASAGNA-Search 2.0 to screen the TF for the targeted gene. After inputting the gene symbol and selecting the species as human, we obtained the name for TF, the binding sequence of our genome, the binding position, strand, binding score, p-value and E-value. Sequence with the maximum binding score at $P < 0.01$ was considered as the binding site of targeted genes.

2.8. Hoechst 33324 staining

The condensation of chromatin in cells is an indicator to cytotoxicity. HepG2 cells were stained with Hoechst-33324 after being incubated with the doses of Tanshinone IIA for 24 h and washed twice with PBS. Cells were then stained with Hoechst-33324 for 20 min at room temperature. The morphology of cells was observed under a Leica fluorescence microscope (Leica, Wetzlar, Germany).

2.9. Kinetic binding analysis

The potential of binding affinity between p53 and PTPN11 was performed on Biomolecular Interaction Analysis Plex Array HT (V3, USA). Twenty eight interested sequences, Rapamycin and Sennoside (set as positive control), and DMSO (negative control) were printed on