Chemico-Biological Interactions 244 (2016) 1–8

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

# The protective effects of paeonol against epirubicin-induced hepatotoxicity in 4T1-tumor bearing mice via inhibition of the PI3K/Akt/NF-kB pathway

Jing Wu [a, b], Xia Xue [a], Bin Zhang [a], Wen Jiang [c], Hongmei Cao [d], Rongmei Wang [a], Deqing Sun [a, *], Ruichen Guo [e, **]

[a] Department of Pharmacy, The Second Hospital of Shandong University, Jinan, China
[b] Department of Pharmacology, School of Medicine, Shandong University, Jinan, China
[c] Central Laboratory, The Second Hospital of Shandong University, Jinan, China
[d] Department of Out-patient, People' Hospital of Zhangqiu, Jinan, China
[e] Institute of Clinical Pharmacology, Qi Lu Hospital of Shandong University, Jinan, China

ARTICLE INFO

*Article history:*
Received 27 August 2015
Received in revised form
9 November 2015
Accepted 23 November 2015
Available online 2 December 2015

*Keywords:*
Epirubicin
Breast cancer
Paeonol
Hepatotoxicity
PI3K/Akt/NF-kB

ABSTRACT

Epirubicin is widely used for the treatment of various breast cancers; however, it has serious adverse side effects, such as hepatotoxicity, which require dose-adjustment or therapy substitution. Paeonol, an active component from Moutan Cortex, has a variety of biological activities, including preventing or reducing various toxicities induced by antineoplastics. Protection by paeonol against hepatotoxicity induced by epirubicin and the underlying mechanism of action were investigated in this study. Cytosolic enzymes in the serum and oxidative stress indices in the liver were determined. The protective effects were determined using the MTT assay *in vitro* or by evaluating the expression of apoptotic factors and crucial proteins in the PI3K/Akt/NF-kB pathway using western blot analysis. It is concluded that paeonol alleviates epirubicin-induced hepatotoxicity in 4T1-tumor bearing mice by inhibiting the PI3K/Akt/NF-kB pathway.



## 1. Introduction

Epirubicin (EPI), a member of the anthracycline family, is widely used to treat breast cancer [1], but it has serious adverse effects, such as cardiotoxicity and hepatic damage, which often requires dose adjustment or therapy substitution [2,3]. Therefore, studies to identify hepatoprotective drugs to protect from EPI-induced liver damage during EPI therapy are a priority. Paeonol (Pae), an active component extracted from the Chinese herbal medicine Moutan Cortex has various biological activities, such as antitumor, antioxidant and anti-inflammatory functions [4,5]. In our previous study, Pae exerted its antioxidative stress activity by affecting nuclear factor-κB (NF-κB) activation involved in cardiomyocyte apoptosis induced by EPI. Furthermore, other studies provide evidence that Pae prevents cisplatin-induced nephrotoxicity by inhibiting nitrosative stress [6]. Phosphatidylinositol-3-kinase (PI3K)/Akt pathway plays an essential role in the protective effects of carbon monoxide and Oleanolic Acid against hepatic Ischemia-Reperfusion injury [14]. Several studies have suggested that EPI-induced hepatotoxicity is also related to oxygen-free radicals produced during EPI metabolism [7]. Moreover, EPI was able to activate the PI3K/Akt pathway, which in turn activates the NF-κB pathway.

In this study, the protective effects against hepatic injury *in vitro* and *in vivo* following EPI exposure via the regulation of the PI3K/Akt/NF-κB signaling pathway were investigated.

## 2. Materials and methods

### 2.1. Reagents and chemicals

Epirubicin (EPI, 10 mg) and paeonol (Pae, purity, 99.12% by HPLC) were purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA.

* Corresponding author. Department of Pharmacy, The Second Hospital of Shandong University, 247# Beiyuan Road, Jinan, 250033, China.
** Corresponding author. Institute of Clinical Pharmacology, Qi Lu Hospital of Shandong University, 44# West Wenhua Road, Jinan, 250012, China.
*E-mail addresses:* sundq0405@126.com (D. Sun), grc7636@126.com (R. Guo).

http://dx.doi.org/10.1016/j.cbi.2015.11.025
0009-2797/

### 2.2. Cell lines [8]

Primary hepatocytes were isolated from mouse livers using the collagenase perfusion methods as follows: livers were perfused with ethylene glycol tetraacetic acid buffer followed by D-Hanks solution containing type I collagenase. Subsequently, the livers were removed, cut into 1 $mm^3$ pieces and filtered through a 100-μm cell strainer (BD Biosciences). Cells were seeded into 6-well plates and cultured in a 5% $CO_2$-jacketed incubator.

Mouse breast tumor cells (4T1) were purchased from Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences (cat number: TCM32). Cancer cells were maintained in RMPI-1640 medium containing 10% FBS and penicillin streptomycin at 37 °C in a humidified 5% $CO_2$-jacketed incubator. Cells >80% confluence were used for experiments.

### 2.3. Cell proliferation assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the proliferation of primary hepatocytes. Cells ($5 \times 10^3$ per well) cultured in 96-well plates were incubated for 48 h with various concentrations of EPI (0.5, 1, 2, 4, and 8 μM) in the presence or absence of Pae (10, 20, 40, and 80 μM). Cell viability was measured at 490 nm. The percentage of cell growth inhibition was calculated as follows: cell inhibition percentage (%) = (1 − mean absorbance in test wells/mean absorbance in control wells) × 100%.

### 2.4. Animals and ethics

Female BALB/c mice (eight weeks, weighting 18–22 g) were purchased from the Laboratory Animal Center, Shandong University, China. The animals were housed in a specified chamber with controlled air conditions (temperature 20–25 °C, humidity 50–65%) and free access to sterile food and water. The protocol for *in vivo* experiments with mice was approved by the Institutional Care and Use Committee of Shandong University (Permit Number: 201402079) and was performed according to the Guide for the Care and Use of Laboratory Animals published by the US National.

### 2.5. Experimental protocol

A 4TI cell suspension (100 μL, $5 \times 10^6$ cells/mL) was injected subcutaneously into the right side of the fourth mammary gland of each mouse. The tumor length (a) and width (b) were measured every other day, and the tumor volume was calculated using the following formula: V ($mm^3$) = ($a \times b^2$) / 2 [9]. When the size of the tumors was approximately 100 $mm^3$, the mice were randomly divided into four groups (n = 8) for treatment as follows:

1. Cont group (n = 8): mice received intragastrically administered (i.g.) saline for three days and a tail intravenous injection of saline on day 4;
2. Pae group (n = 8): mice received i.g. Pae (20 mg/kg) for three days and a tail intravenous injection of saline on day 4;
3. EPI group (n = 8): mice received i.g. saline for three days and a tail intravenous injection of EPI (9 mg/kg) on day 4;
4. Pae + EPI group (n = 8): mice received i.g. Pae (20 mg/kg) for three days and a tail intravenous injection of EPI (9 mg/kg) on day 4.

Blood was collected and the mice were sacrificed after 2 days. The livers were removed rapidly and weighed. Several tissues were fixed in formalin for hematoxylin-eosin (H&E) staining and terminal deoxynucleotidyl transferase mediated dUDP nick end-labeling (TUNEL) staining, whereas others were rapidly frozen in liquid nitrogen and stored at −80 °C for western blotting (WB) analysis.

### 2.6. Measurement of body and liver weights

The body weights of all of the mice were recorded twice a day during the experiment, and the livers were removed and measured in grams per gram of body weight.

### 2.7. Assay of hepatic marker enzymes

A biochemistry index in mouse blood was monitored. Before the mice were sacrificed, blood was collected into a centrifuge tube containing heparin after 2 days of treatment, and the plasma was obtained after centrifugation at 4000 rpm for 5 min. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) activities in the plasma were assayed according to the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China).

### 2.8. Estimation of MDA, GSH, SOD and CAT

The liver samples were homogenized at 4 °C and centrifuged at 1500 rpm for 30 min. Then, the supernatants were obtained and used to measure the content of malondialdehyde (MDA) and glutathione (GSH) and the activity of superoxide dismutase (SOD) and catalase (CAT) according to the manufacturer's protocols (Nanjing Jiancheng Biotechnology Institute, China).

### 2.9. Histopathological examination and TUNEL staining

The histopathological examination of the mouse livers was as follows: the liver tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned (4–5 mm thick), stained with HE, and examined under a bright-field microscope (CKX41, 170 Olympus, Tokyo, Japan) to observe general histological features. For the analysis of cell death, apoptotic cells were labeled using TUNEL staining (Roche, USA). All procedures followed the manufacturer's instruction. The apoptotic cells were identified by dark brown nuclear staining counted with 100 cells from six random microscopic fields in each group, and the values were represented as percentage of the total number of cells.

### 2.10. RNA preparation and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from Balb/c mice livers using the TRIZOL reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The cDNA synthesis was performed from 2 μg RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The expression of IL-2, IL-6, TNF-α and IFN-γ was characterized by Reverse transcription polymerase chain reaction (RT-PCR) using the LightCycler from Roche Diagnostics (Mannheim, Germany). The expression levels of The target genes were normalized to β-actin. The sequences of the PCR primers are shown as follows: IL-2, Forward-5′-CCTGAGCAGGGAGAATTACA-3′, Reverse-5′-TCCAGAACATGCCGCAGA-3′; IL-6, Forward-5′-GCTACCAAACTGGATATAATCAGGA-3′, Reverse-5′-CCAGGTAGCTATGGTACTCCAGAA-3′; TNF-α, Forward-5′-CTGTAGCCCACGTCGTAGC-3′, Reverse-5′-TTGAGATCCATGCCGTTG-3′; IFN-γ, Forward-5′-ATCTGGAGGAACTGGCAAAA-3′, Reverse-5′-TTCAAGACTTCAAAGAGTCTGAGG-3′, β-actin, Forward-5′-GAAATCGTGCGTGACATCAAA G-3′, Reverse-5′-TGTAGTTTCATGGATGCCACA G-3′ [10,11].