Phytomedicine 24 (2017) 134–140

Contents lists available at ScienceDirect

Phytomedicine

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

Original article

Identification of acetylshikonin as the novel CYP2J2 inhibitor with anti-cancer activity in HepG2 cells

See-Hyoung Park[a,1], Nguyen Minh Phuc[b,1], Jongsung Lee[c,1], Zhexue Wu[b], Jieun Kim[b], Hyunkyoung Kim[b,d], Nam doo Kim[d], Taeho Lee[b], Kyung-Sik Song[b,*], Kwang-Hyeon Liu[b,*]

[a] Department of Bio and Chemical Engineering, Hongik University, Sejong 30016, Republic of Korea
[b] BK21 Plus KNU Multi-Omics based Creative Drug Research Team, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 41566, Republic of Korea
[c] Department of Genetic Engineering, Sungkyunkwan University, Suwon 16419, Republic of Korea
[d] New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu 41061, Republic of Korea

ARTICLE INFO

Article history:
Received 7 June 2016
Revised 28 November 2016
Accepted 1 December 2016

Keywords:
Acetylshikonin
CYP2J2
Inhibition
Anti-cancer

ABSTRACT

Background: Acetylshikonin is one of the biologically active compounds derived from the root of *Lithospermum erythrorhizon*, a medicinal plant with anti-cancer and anti-inflammation activity. Although there have been a few previous reports demonstrating that acetylshikonin exerts anti-cancer activity in vitro and in vivo, it is still not clear what is the exact molecular target protein of acetylshikonin in cancer cells.
Purpose: The purpose of this study is to evaluate the inhibitory effect of acetylshikonin against CYP2J2 enzyme which is predominantly expressed in human tumor tissues and carcinoma cell lines.
Study design: The inhibitory effect of acetylshikonin on the activities of CYP2J2-mediated metabolism were investigated using human liver microsomes (HLMs), and its cytotoxicity against human hepatoma HepG2 cells was also evaluated.
Method: Astemizole, a representative CYP2J2 probe substrate, was incubated in HLMs in the presence or absence of acetylshikonin. After incubation, the samples were analyzed by liquid chromatography and triple quadrupole mass spectrometry. The anti-cancer activity of acetylshikonin was evaluated on human hepatocellular carcinoma HepG2 cells. WST-1, cell counting, and colony formation assays were further adopted for the estimation of the growth rate of HepG2 cells treated with acetylshikonin.
Results: Acetylshikonin inhibited CYP2J2-mediated astemizole *O*-demethylation activity (K_i = 2.1 μM) in a noncompetitive manner. The noncompetitive inhibitory effect of acetylshikonin on CYP2J2 enzyme was also demonstrated using this 3D structure, which showed different binding location of astemizole and acetylshikonin in CYP2J2 model. It showed cytotoxic effects against human hepatoma HepG2 cells (IC_{50} = 2 μM). In addition, acetylshikonin treatment inhibited growth of human hepatocellular carcinoma HepG2 cells leading to apoptosis accompanied with p53, bax, and caspase3 activation as well as bcl2 down-regulation.
Conclusion: Taken together, our present study elucidates acetylshikonin displays the inhibitory effects against CYP2J2 in HLMs and anti-cancer activity in human hepatocellular carcinoma HepG2 cells.

Introduction

Hepatocellular carcinoma (HCC) is the main subtype cancer among liver cancer that worldwide is the third cause of the cancer-initiated death in human (Jemal et al., 2011; Libby et al., 2014; Padhya et al., 2013). The first treatment option for HCC is the surgical resection. However, only early stage HCC patients can be cured by this procedure (Cheng and Lv, 2013; Uhl et al., 2014). In addition, recent diagnostic technique for HCC often fails to detect the early stage HCC (Padhya et al., 2013). So far, sorafenib, one of receptor tyrosine kinase inhibitors, is the only drugs approved by Food and Drugs Administration (FDA) and has been used for the systematic treatment of HCC (Reataza and Imagawa, 2014). Lots of candidate chemotherapeutic agents have been promisingly developed for the patients who cannot be cured by the surgical

Abbreviations: HLMs, human liver microsomes; HCC, Hepatocellular carcinoma; DMSO, dimethyl sulfoxide; G6P, glucose-6-phosphate; G6PDH, G6P dehydrogenase; $NADP^+$, β-nicotinamide dinucleotide phosphate; EETs, Epoxyeicosatrienoic acids.

* Corresponding authors. Fax: +82 53 950 8557.
E-mail addresses: kssong@knu.ac.kr (K.-S. Song), dstlkh@knu.ac.kr (K.-H. Liu).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.phymed.2016.12.001
0944-7113/

resection as well as sorafenib treatment (Cheng and Lv, 2013; Loong and Yeo, 2014; Reataza and Imagawa, 2014). However, it is still necessary to find more potent chemotherapeutics that has less side-effect and reduces the drug resistance in the present chemotherapy (Holohan et al., 2013; Kratz et al., 2008)).

Acetylshikonin is one of the naturally occurring derivative forms of shikonin that are usually found in the root of Lithospermum erythrorhizon Siebold et Zucc. (Xiong et al., 2009). Traditionally, acetylshikonin is used for inflammation-related infected wounds (Lu et al., 2008; Wang et al., 1997). Recently, several reports have demonstrated that acetylshikonin have anti-tumor activities such as inhibition of tumor angiogenesis (Pietrosiuk et al., 2004) and induction of apoptosis on melanoma cell (Kretschmer et al., 2012). However, it is not fully understood about the exact working mechanism of acetylshikonin on anti-tumor activity in HCC.

In this study, we identified acetylshikonin as new CYP2J2 enzyme inhibitor. It suppresses cell proliferation and induces apoptosis in human hepatocellular carcinoma Hep2 cells.

Materials and methods

Materials

Mouse anti-β-actin antibody and the following chemicals and solvents (dimethyl sulfoxide (DMSO), glucose-6-phosphate (G6P), G6P dehydrogenase (G6PDH), glycerol, glycine, mebendazole, β-nicotinamide dinucleotide phosphate ($NADP^+$), sodium chloride, Trizma base, and Tween 20) were from Sigma (St. Louis, MO). Astemizole and *O*-desmethylastemizole were purchased from Toronto Research Chemicals (North York, Canada). Acetylshikonin (>95.0%) were a gift from the Institute for Korea Traditional Medical Industry (Daegu, Korea). Pooled human liver microsomes (HLMs, H0630) were purchased from XenoTech (Lenexa, KS). Recombinant CYP2J2 isoform was obtained from Corning Life Sciences (Corning, NY). Rabbit anti-CYP2J2, rabbit anti-p-p53(Ser15), rabbit anti-p-p53(Ser20), rabbit anti-p-p53(Ser46), mouse anti-PARP1, rabbit anti-p-Akt, rabbit anti-Akt, rabbit anti-p-Erk, rabbit anti-Erk, rabbit anti-p-Jnk, rabbit anti-Jnk, and mouse anti-p53 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cleaved caspase3, rabbit anti-p21, rabbit anti-Bax, rabbit anti-cleaved PARP1, and rabbit anti-Bcl2 antibody were from Cell Signaling (Danvers, MA). Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG were obtained from Jackson ImmunoResearch (West Grove, PA). ECL Western Blotting Detection Reagents were obtained from Genedepot (Barker, TX).

CYP2J2 activity assay

The incubation mixtures containing pooled HLMs (XenoTech, 0.1 mg/ml), astemizole (1 μM), and acetylshikonin (0, 0.2, 0.5, 1, 2, 5, or 20 μM) were preincubated at 37 °C for 5 min. The final concentration of DMSO in the incubation mixtures was 1.0% (v/v). The reaction was initiated by addition of NADPH-generating system (1.3 mM $NADP^+$, 3.3 mM G6P, 3.3 mM magnesium chloride, and 500 units/ml G6PDH), and terminated by the addition of 100 μl of acetonitrile containing 300 nM mebendazole into the incubation mixtures after 15 min. After vortexing and centrifuging at 13,000 $\times g$ for 5 min, aliquots of the supernatant (1 μl) were analyzed by liquid chromatography-tandem mass spectrometry as described previously (Lee et al., 2014). To determine the inhibitory potential of acetylshikonin against CYP2J2-catalyzed astemizole *O*-demethylase activity in HLMs, acetylshikonin (0, 0.5, 2, 5, or 10 μM) was added to reaction mixtures containing different concentrations of astemizole (0.2, 1, or 5 μM).

Molecular docking

As there is no crystal structure data available in the protein structure database, we constructed CYP2J2 model structure. For this, we adopted the crystal structure of human CYP2C8 (pdb code: 2NNI) as the template (Schoch et al., 2008). The Discovery Studio 2016 was used for homology model construction (Biovia, San Diego). Sequence analysis identified homologs for CYP2J2 sequences by BLAST search using NCBI server. Finally, the modeled structure of CYP2J2 was refined by energy minimization for molecular docking. The starting coordination of the CYP2J2 was further modified for docking calculation. The protein structure was minimized using the Protein Preparation Wizard by applying an OPLS2005 force field (Sastry et al., 2013). For the binding model prediction of astemizole and acetylshikonin on CYP2J2, the initial structure was built using Maestro build panel and energy was minimized using the MacroModel module in the Schrödinger software package (version 11.0, Schrödinger). Schrödinger QM-Polarized Ligand Docking Protocol (QPLD) was performed the docking model of astemizole and acetylshikonin with CYP2J2 (Cho et al., 2005).

WST-1 cell viability assay

Human hepatocellular carcinoma HepG2 cells and human normal hepatocytes (from ATCC) were maintained in EMEM supplemented with 10% fetal bovine serum and 1% Streptomycin/Penicillin at 37 °C in a humidified incubator containing 5% CO_2 in air. A 200 μl aliquot of cells (1×10^3 cells in media) was added to 96 well plate and incubated for 18 h at 37 °C in a humidified incubator containing 5% CO_2 in air. After incubation, each dose (0, 2.5, 5, 10, 20, or 40 μM) of each chemicals was added into each well for 48 h for the dose-dependent assay. Control cultures were treated with DMSO. After incubation, a 20 μl WST-1 solution (Daeillab Service, Korea) was added to each well and the incubation continued for 4 h The visible absorbance at 460 nm of each well was quantified using a microplate reader.

Cell counting assay

Cells (1×10^4) were seeded in 6 cm dishes and incubated at 37 °C in a humidified incubator containing 5% CO_2 in air incubator for 18 h After incubation, cells were treated with DMSO as control vehicle and the indicated concentration of acetylshikonin (500 nM) for 0, 24, 72, or 120 h At each day, cell numbers were measured by using Hemocytometer.

Colony formation assay

Cells (0.5×10^3) were seeded in 6 cm dishes and incubated at 37 °C in a humidified incubator containing 5% CO_2 in air incubator for 18 h After incubation, cells were treated with DMSO as control vehicle and the indicated concentration of acetylshikonin (500 nM) for 14 days. And then the colonies were washed twice with PBS, fixed with 3.7% paraformaldehyde, and stained with 1% crystal violet solution in distilled water.

Western blotting

Cells were washed with PBS and lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, pH 8.0) with protease and phosphatase inhibitors. Cell lysates were centrifuged (10,000 $\times g$, 4 °C, 10 min) and the supernatants were separated on 6% or 10% SDS–PAGE gels and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in 3% non-fat dry milk for 1 h at room temperature, and probed with appropriate antibodies. Membranes