Toxicology Letters 281 (2017) 74–83

Contents lists available at ScienceDirect

Toxicology Letters

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

Full length article

Assessment of the inhibition risk of shikonin on cytochrome P450 via cocktail inhibition assay

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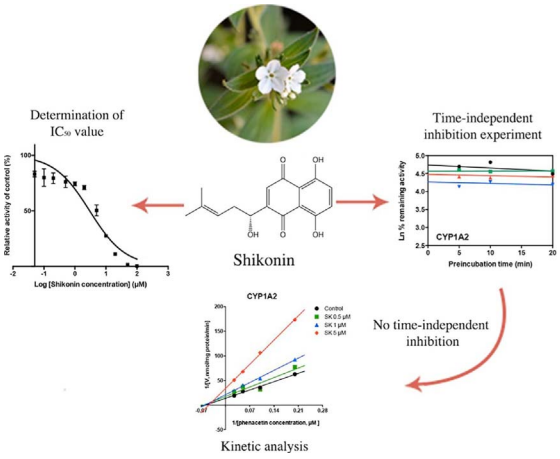
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Cytochrome P450
Drug-drug interactions
Food-drug interactions
Shikonin
Cocktail approach

ABSTRACT

Shikonin is a naphthoquinone pigment extracted from roots of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), and possesses various pharmaceutical activities, such as anti-inflammation and anti-cancer effects. In addition, shikonin as a natural red colorant for food garnishment and cosmetics ingredient is widely used in the world. However, the inhibition risk of shikonin on cytochrome P450 (CYP) remains unclear. The aim of this study was to investigate the potential inhibition of shikonin against CYP1A2, CYP2B1/6, CYP2C9/11, CYP2D1/6, CYP2E1 and CYP3A2/4 activities in human and rat liver microsomes through cocktail approach *in vitro*. The results demonstrated that shikonin exhibited no time-dependent inhibition of CYP activities. In human liver microsomes, shikonin was not only a mixed inhibitor of CYP1A2, CYP2B6, CYP2C9, CYP2D6 and CYP3A4, but also a competitive inhibitor of CYP2E1, with K_i values no more than 7.72 μM. In rat liver microsomes, shikonin also exhibited the mixed inhibition on CYP1A2, CYP2B1, CYP2C11, CYP2D1, and the competitive inhibition on CYP2E1. Interestingly, shikonin presented an atypical kinetic inhibition of CYP3A2-mediated midazolam 1-hydroxylation in rats. In conclusion, the relatively low K_i values of shikonin would have a high risk

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http://dx.doi.org/10.1016/j.toxlet.2017.09.014
Received 23 May 2017; Received in revised form 18 August 2017; Accepted 19 September 2017
Available online 21 September 2017
0378-4274/

potential to cause the possible toxicity, especially drug-drug or food-drug interactions based on the potent inhibition of CYP enzymes.

1. Introduction

Lithospermum erythrorhizon Sieb. et Zucc., known as Zicao, is documented in the "Bencao Gangmu" as an extensively-used Traditional Chinese Medicine for treating inflammation diseases in China. Shikonin (5,8-dihydroxy-2-[(1R)-1-hydroxy- 4-methyl-3-pentenyl]-1,4- naphthoquinone, Fig. 1) and its derivatives isolated from dried roots of *Lithospermum* are the principle components responsible for possessing therapeutic effects. With the exponentially increasing attention on shikonin, the major pharmacological properties of shikonin have been already elaborated in the previous studies (Andujar et al., 2013a), such as anti-inflammatory (Lee et al., 2010), antioxidant (Jin and Bai, 2012), antimicrobial (Miao et al., 2012) and wound healing activities (Andujar et al., 2013b).

In recent years, many studies have reported that shikonin exhibits potential anti-tumor effects on different kinds of cancers, such as myelogenous leukemia (Mao et al., 2008), breast cancer (Yin et al., 2016) and hepatocellular carcinoma (Song et al., 2016) through multiply molecular targets, thus suggesting that shikonin is a promising candidate for the development of antineoplastic drug. Moreover, in East Asia, especially in China, Japan and Korea, shikonin is specifically used as a colorant for food (Albreht et al., 2012; Ito et al., 2011; Kim et al., 2015) or a beneficial nature compound for cosmetics (Kim et al., 2015; Lee et al., 2008). For example, shikonin together with other components of *Lithospermum* are applied as pigments named "gromwell red", which are widely used in China as an additive for food and beverage.

Cytochrome P450 (CYP) enzymes are the major Phase I enzymes responsible for biotransformation of most drugs and other hydrophilic xenobiotics. In addition, CYP enzymes participate in cellular function through biosynthesis and metabolism of endogenous molecules (Nebert and Dalton, 2006). Actually, induction or inhibition of CYP will cause variation in drug pharmacokinetics, leading to decrease pharmacological efficacy or enhance the toxicity (Wang et al., 2010). Therefore, the U.S. Food and Drug Administration (FDA) has recommended investigating potential CYP inhibition of co-administration drugs, herbs or even food, which may avoid unnecessary attritions in drug development (Kerns and Di 2003; Sun et al., 2014). In particular, there is no related investigation between shikonin and CYP enzymes despite of its long-term application for Chinese herbal decoction and food garnishment.

The purpose of present study was to evaluate the inhibitory effects of shikonin on the major CYP enzymes, including CYP1A2, CYP2B1/6, CYP2C9/11, CYP2D1/6, CYP2E1, CYP3A2/4, in both human and rat liver microsomes via a rapid six-in-one cocktail approach, which is a powerful tool for high-throughput inhibition risk screening *in vitro* (Dinger et al., 2014; Spaggiari et al., 2016). Enzyme inhibition kinetic analysis were also performed to study the mode of inhibition of shikonin on different CYP isoforms in human and rat liver microsomes using the CYP probe substrates.

2. Materials and methods

2.1. Chemicals and reagents

Shikonin (purity > 98%) was purchased from Dalian Meilun Biotech Co., Ltd (Dalian, China). Phenacetin, bupropion, tolbutamide, dextromethorphan, midazolam, 3-acetamidophenol (internal standard), chlorpropamide (internal standard), glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), β-nicotinamide adenine dinucleotide phosphate (NADP) and tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chlorzoxazone was purchased from Alfa Aesar (Massachusetts, USA). 4-acetamidophenol, hydroxybupropion, 4-hydroxytolbutamide, dextrorphan, 6-hydroxychlorzoxazone and 1-hydroxymidazolam were obtained from Toronto Research Chemical (North York, Canada). Mebendazole (internal standard) was obtained from Aladdin Industrial Co. (California, USA). Pooled human liver microsomes (HLMs, n = 20) were obtained from Corning Gentest Corporation (Woburn, MA, USA) and stored at −150 °C until use. All the experimental procedures involving humans have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) guidelines. Acetonitrile and methanol (all HPLC grade) were obtained from Fisher Chemicals (Leicester, UK). Formic acid (HPLC grade) was purchased from TEDIA (Ohio, USA). Distilled water was purified in a Millipore system Milli Q (Millipore Corp., Bedford, MA, USA).

2.2. Animals

Male Sprague-Dawley rats (200–250 g) were supplied by National Rodent Laboratory Animal Resources, Shanghai Branch of China. Rats were kept in a specific pathogen-free facility and fed with standard food and water with 12 h light-dark cycles. All the methods in animals were carried out in accordance with the National Institutes of Health standards established in the 'Guidelines for the Care and Use of Experimental Animals'. All experimental protocols in animals were approved by the Ethics Committee on Animal Experimentation of East China Normal University (Shanghai, China).

2.3. Preparation of rat liver microsomes

The animals were fasted overnight and killed by cervical dislocation before removal of the liver. The liver was excised, rinsed with ice-cold saline (0.9% NaCl w/v), weighed and homogenized in a 0.05 M Tris/KCl buffer (pH 7.4). The homogenate was centrifuged at 10,500 × *g* at 4 °C for 20 min, and the supernatant was centrifuged at 105,000 × *g* at 4 °C for 60 min. Then, the supernatant was discarded, and precipitate was further centrifuged at 105,000 × *g* at 4 °C for 60 min. The pellet was reconstituted with 0.05 M Tris/KCl buffer (pH 7.4) and stored at −150 °C until use. The protein concentration of the rat liver microsomes (RLMs) was determined by a protein quantitative assay using bicinchoninic acid (Sun et al., 2014).

2.4. Assays of CYP enzymes activities

According to the guideline of FDA, the activities of CYP enzymes were assessed by the formation of 4-acetamidophenol from Phenacetin (CYP1A2), hydroxybupropion from bupropion (CYP2B1/6), 4-hydroxytolbutamide from tolbutamide (CYP2C6/11), dextrorphan from

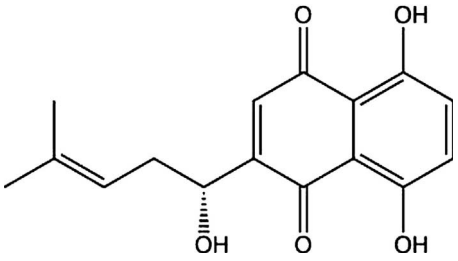

Fig. 1. Chemical structure of shikonin.