Original Article
Drug-drug interaction potential of 1,5-dicaffeylquinic acid, a new herbal drug for the treatment of hepatitis B and human immunodeficiency virus infection, based on the inhibition of cytochrome P450s in human and rat liver microsomes

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Abstract: The inhibition of cytochrome P450s (CYPs) is regarded as one of the most important causes for drug-drug interactions. 1,5-Dicaffeylquinic acid (1,5-DCQA) is currently being evaluated in a phase II clinical study in China for the treatment of hepatitis B and human immunodeficiency virus infections. The purpose of this study was to investigate the *in vitro* inhibitory effect of 1,5-DCQA on six major CYP enzymes to assess its safety through its potential to interact with co-administered drugs. Seven CYP probe substrate metabolites (acetaminophen for CYP1A2, 6α-hydroxypaclitaxel for CYP2C8, 4-hydroxydiclofenac for CYP2C9, 4-hydroxymephenytoin for CYP2C19, dextrorphan for CYP2D6, and 6β-hydroxytestosterone and 1-hydroxymidazolam for CYP3A4) were measured simultaneously by LC-MS/MS. 1,5-DCQA was incubated with human and rat liver microsomes in the presence of seven CYP450 isoform substrates, and the *in vitro* inhibitory effects were evaluated by determining the IC_{50} values. 1,5-DCQA showed negligible inhibitory effects on the six major human (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) and rat CYP isozymes (Cyp1a2, Cyp2c7, Cyp2c11, Cyp2c79, Cyp2d4, and Cyp3a2). All IC_{50} values exceeded 100 µM. Our study demonstrates that 1,5-DCQA is unlikely to cause significant drug-drug interactions in humans when co-administered with drugs metabolized by the six CYP isozymes.

Keywords: 1,5-dicaffeylquinic acid, cytochrome P450, human/rat liver microsomes, drug-drug interactions

Introduction

The hepatitis B virus (HBV), influenza A and B viruses, human immunodeficiency virus (HIV), and most hemorrhagic fever viruses, infect humans with high incidence and mortality rates, are a serious threat to human health, and have caused inestimable social and economic damage. According to the Global Burden of Disease Study 2010, the annual numbers of deaths from HBV and HIV infection are estimated at 786,000 and 1,470,000, respectively, ranking 15th and 6th, respectively, among the total causes of death [1]. At present, at least 6 drugs in two main classes have been approved for clinical use in the treatment of HBV infections, and about 36 drugs in five main classes

have been approved for the treatment of HIV infection/AIDS [2, 3]. However, all the approved therapeutic drugs for HBV and HIV have limitations and are far from satisfactory owing to their low specificity, side effects, and high rates of drug resistance. Thus, there exists a significant unmet medical need for new, safe, and effective drugs.

Natural products, particularly those used in traditional medicine, have a broad potential in the treatment of viral infectious diseases owing to their obvious advantages including rich resources, low price, and fewer adverse effects. Accordingly, it is essential to evaluate the safety of traditional medicines, particularly medicinal plants and other natural products that may
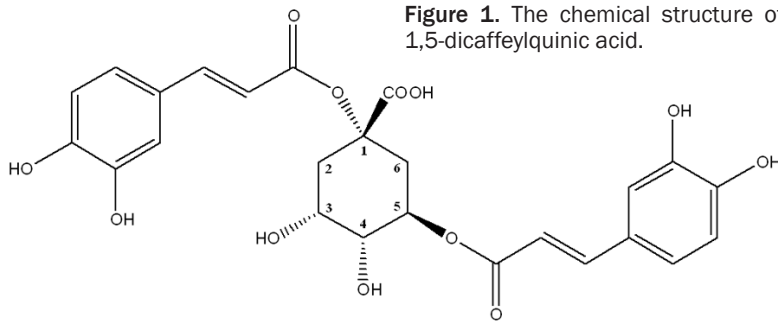
Figure 1. The chemical structure of 1,5-dicaffeylquinic acid.

yield effective and affordable therapeutic agents.

In recent years, numerous medicinal plants (such as Flos Lonicerae, Rhizoma Polygoni Cuspidati, and Fructus Crotonis), active compounds (for example, quercetin, saikosaponin, and baicalein), and Chinese medicinal compounds (for example, Banlangen granules, Liujunzi decoction, and Liuwei Dihuang Wan) were reported to exhibit potent anti-HBV and/or anti-HIV effects [4-7]. Dicaffeoylquinic acids (DCQAs) are a class of natural phenolic acid compounds characterized by two caffeic acid molecules connected to one quinic acid molecule through ester bonds. DCQAs are distributed widely in many kinds of traditional medicinal plants including Compositae, Leguminosae, Umbelliferae, Caprifoliaceae, and Convolvulaceae. DCQAs have recently drawn increasing attention for their anti-HIV activity. In addition, DCQAs show a multitude of significant pharmacological activities such as anti-oxidation, anti-inflammation, anti-HBV, anti-immunosuppressive, hepatoprotective, and neuroprotective effects [8, 9].

Of the known DCQAs, 1,5-dicaffeylquinic acid (1,5-DCQA, **Figure 1**) has gained increasing attention in the biomedical community in recent years. It possesses a broad spectrum of pharmacological properties such as anti-oxidation [10, 11], anti-inflammation [12-14], anti-HIV [15, 16], hepatoprotective [17-20], and neuroprotective effects [21-25]. Because various studies indicated that 1,5-DCQA is a selective inhibitor of HIV-1 integrase and possessed hepatoprotective activities, Dong et al. applied for patents (China patent number: ZL 96111691.9; European patent number: EP 1008344B1; US patent number: US 6331546B1). 1,5-DCQA was evaluated in a phase I clinical study in

2006, then a phase II clinical study in 2010. The China Food and Drug Administration has approved it as a promising novel drug for the treatment of HIV and HBV infections.

1,5-DCQA, as a novel anti-HIV and anti-HBV drug, may soon be available for combination therapy in the clinic as its safety and pharmacokinetic drug-drug interaction properties are being evaluated. One study suggested that O-methylation and glucuronidation were two important metabolic pathways of 1,5-DCQA in both rat liver and small intestine, and that the HIV-1 inhibitory activities of 1,5-DCQA metabolites were comparable to or slightly weaker than those of 1,5-DCQA itself [26]. Another study showed that O-methylation and glucuronidation of 1,5-DCQA were two factors causing its rapid elimination from the circulation in rats [27]. Further research in humans suggested that O-methylation, glucuronidation, and isomerization were three important metabolic pathways of 1,5-DCQA [28]. Phase I clinical trials with 1,5-DCQA tablets indicated that single dose regimens up to 1200 mg, and multiple dose regimens up to 500 mg, twice daily, were safe and tolerable in Chinese healthy volunteers [29, 30].

Any drug may alter the absorption, distribution, metabolism, and excretion of a co-administered drug resulting in increased or decreased plasma concentrations that can lead to serious adverse events or reduced drug efficacy. Inhibition of cytochrome P450 (CYP) isozymes by co-administered drugs is one of the most common reasons for harmful drug-drug interactions. Such interactions have led to the removal of several drugs (e.g., terfenadine, mibefradil, and bromfenac) from the market [31]. The CYP superfamily, one of the most important drug-metabolizing enzyme systems in humans, is responsible for the metabolism of a variety of endogenous compounds (i.e., steroids) and xenobiotics (i.e., drugs) [32]. The CYP 1~3 isoform families are responsible for the metabolism of the vast majority of clinical drugs. Of these drugs, approximately 90% are metabolized by CYP1A2, 2C8/9, 2C19, 2D6, or 3A4/5 [33].

Despite the well-documented and widespread pharmacological studies of 1,5-DCQA, little is known about its potential effects on CYP activities. Only one much earlier paper reported that oral 1,5-DCQA had no effect on the total protein concentration of rat liver CYPs [34]. Thus, it is important to assess the potential inhibitory effects of 1,5-DCQA on specific CYPs with the aim of avoiding pharmacokinetic drug-drug interactions. In the present study, we evaluated systematically the *in vitro* inhibitory potential of 1,5-DCQA on the activities of six major CYPs (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D-6, and CYP3A4) based on the drug interaction guidelines of Food and Drug Administration (FDA), which was worthy of promoting safety and efficacy of 1,5-DCQA in the clinic.

Materials and methods

Chemicals and reagents

The 1,5-DCQA standard was purchased from Shanghai Tauto Biotech (Shanghai, People's Republic of China). The purity of all standards exceeded 98%. Phenacetine, diclofenac, midazolam, dextromethorphan, acetaminophen, furafylline, quinidine, 1-hydroxymidazolam, dextrorphan, sulfaphenazole, gemfibrozil, and buspirone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Paclitaxel, S-mephenytoin, 4-hydroxymephenytoin, S-(+)-N-benzylnirvanol, montelukast, and ketoconazole were purchased from Toronto Research Chemicals (Toronto, Canada). Testosterone was purchased from Acros (Geel, Belgium). 6β-Hydroxytestosterone was purchased from International Laboratory (San Bruno, CA, USA). 6α-Hydroxy-paclitaxel was purchased from Calbiochem (San Diego, CA, USA). 4-Hydroxydiclofenac was purchased from BD Gentest (Woburn, MA, USA). Glucose-6-phosphate and NADP+ were purchased from Majorbio (Shanghai, People's Republic of China), and glucose-6-phosphate dehydrogenase was purchased from Calbiochem (Gibbstown, NJ, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) and used throughout the study. All inorganic salts were of analytical grade and purchased from Sinopharm Chemical Reagent (Shanghai, People's Republic of China). All organic solvents were of HPLC grade and purchased from Sigma-Aldrich.

Pooled human liver microsomes (lot 32556) were purchased from BD Gentest. Pooled rat liver microsomes were prepared from three male Sprague-Dawley rats purchased from HD Biosciences (Shanghai, People's Republic of China).

Microsomal incubations

Incubation mixtures were prepared in a total volume of 60 μL with final component concentrations as follows: 0.1 M potassium phosphate buffer (pH 7.4), 1.0 mM NADP+, 4.0 mM MgCl$_2$, 10 mM glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, 0.3 mg/mL human liver microsomes or 1.0 mg/mL rat liver microsomes, 1,5-DCQA, positive controls, and specific substrates (**Table 1**). Substrates were used at final concentrations approximately equal to their respective Michaelis-Menten constant (Km) values (**Table 1**). NADP+ was added after a 15 min preincubation of all other components at 37°C. After a 30 min incubation, the reactions were terminated by adding 60 μL ice-cold acetonitrile containing internal standards. Incubated samples were stored at -80°C- until liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. The mixture was centrifuged at 3500 rpm for 5 min. The supernatant was mixed with an equal volume of a methanol/water mixture (1:1), and 20 μL of the final mixture was injected for LC-MS/MS analysis.

CYP inhibition assay

A Shimadzu (Tokyo, Japan) LC-20A liquid chromatographic system equipped with a DGL-20A vacuum degasser, a dual pump, and a SIL-20A autosampler was used. Detection was performed on an API 4000 mass spectrometer equipped with a TurboIonSpray (electrospray ionization (ESI)) Interface (Applied Biosystems, Concord, ON, Canada). Analyst 1.5 software packages (Applied Biosystems) were used to control the LC-MS/MS system, as well as for data acquisition and processing.

LC-MS/MS was performed using the method described previously [35, 36]. Chromatographic separation was achieved on a Waters Nova-Pak® C$_{18}$ (150 × 3.9 mm) column. The column temperature was maintained at 25°C. A post-column diverter valve was used to direct the HPLC column elute to a waste container for the first 3.2 min of the chromatographic run, and then to the ionization source. The flow rate was maintained at 0.7 mL/min and used the follow-

Table 1. Incubation conditions and analytical parameters for the individual metabolites and internal standards

Isoform	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4	CYP3A4	IS01	IS02
Substrate	Phenacetin	Paclitaxel	Diclofenac	S-Mephenytoin	Dextromethorphan	Midazolam	Testosterone		
Final concentration (μM)	10	5	5	200	5	2	50		
Metabolite	Acetaminophen	6α-Hydroxypaclitaxel	4-Hydroxydiclofenac	4-Hydroxymephenytoin	Dextrorphan	6β-Hydroxytestosterone	1-Hydroxymidazolam	Buspirone	Gemfibrozil
Retention time (min)	4.0	5.62	5.54	4.95	5.64	5.24	5.85	6.43	6.58
Ionization mode	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Negative
Precursor ion (m/z)	152.0	870.1	309.7	233.2	258.1	305	342	386.2	249.1
Quantifier (m/z)	110.1	286.3, 525.6	266	189.9	157	269	324	122.4	120.9
Inhibitor	Furafylline	Montelukast	Sulfaphenazole	S-(+)-N-3-Benzylnirvanol	Quinidine	Ketoconazole	Ketoconazole		
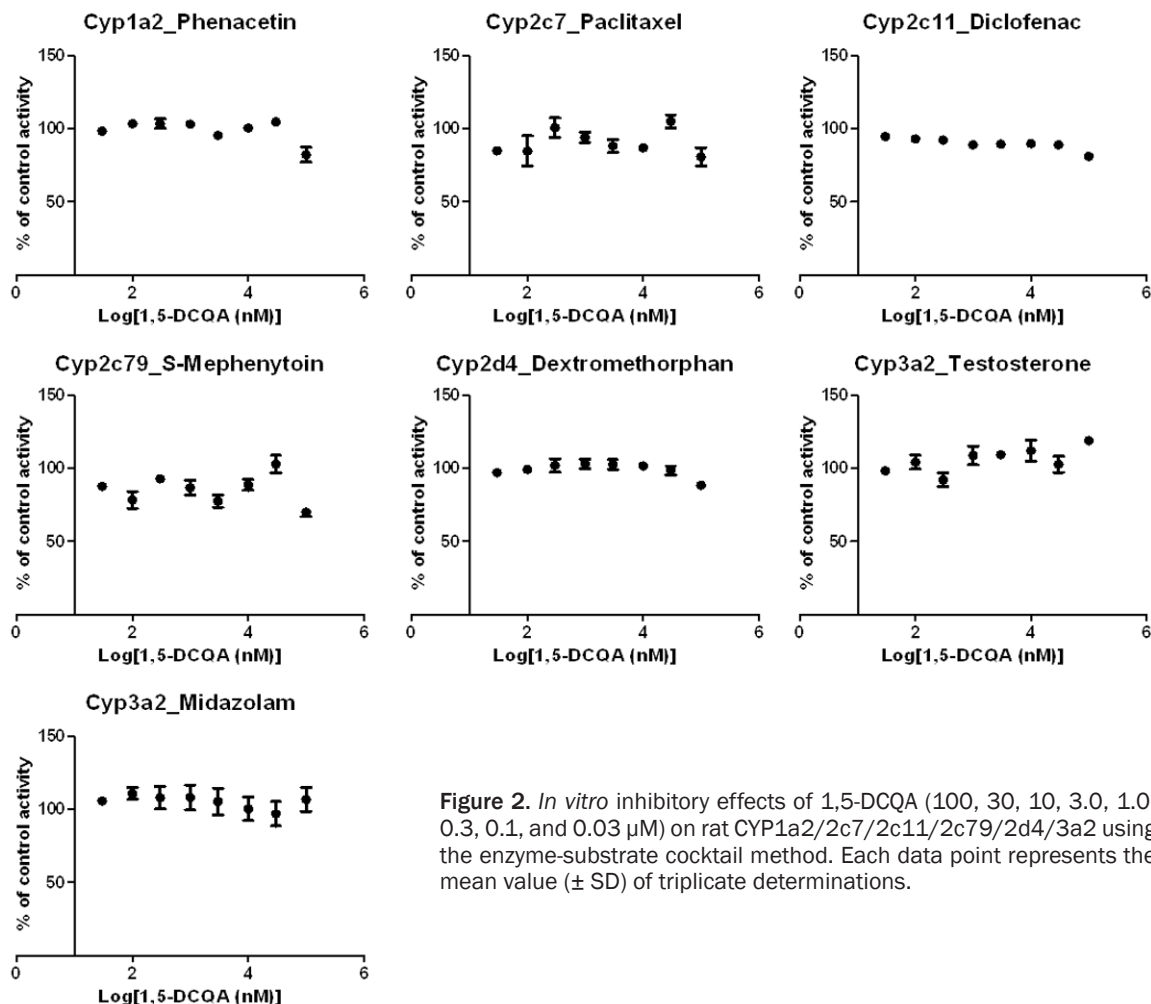
Figure 2. *In vitro* inhibitory effects of 1,5-DCQA (100, 30, 10, 3.0, 1.0, 0.3, 0.1, and 0.03 µM) on rat CYP1a2/2c7/2c11/2c79/2d4/3a2 using the enzyme-substrate cocktail method. Each data point represents the mean value (± SD) of triplicate determinations.

ing mobile phases: solvent A [a mixture of acetonitrile/methanol/formic acid (50:50:0.1)] and solvent B (5 mM ammonium acetate).

The HPLC gradient program was as follows: (1) mobile phase B was 5% at 0 min, (2) a linear gradient was run to 15% B at 2.0 min, (3) a linear gradient was run to 80% B at 3.3 min, (4) a linear gradient was run to 90% B at 3.6 min, (5) an isocratic elution was run at 90% B from 3.6 to 4.0 min, (6) a linear gradient was run to 15% B at 7.0 min, (7) solvent composition was returned to 5% B in 0.1 min for re-equilibration for 2 min.

Mass transitions for the probe substrate metabolites, using the API4000 LC-MS/MS, are listed in **Table 1**. The ESI (+) conditions were 5500 spray voltage, capillary temperature 400°C, sheath gas nitrogen flow 50 psi. The ESI (-) conditions were identical to the ESI (+)

conditions except that a 4500 capillary voltage was used.

Data analysis

Peak area ratios of the metabolites and internal standard were acquired using Analyst 1.5 software (Applied Biosystems). The peak area ratios were plotted as a percentage of the relevant negative control for each reaction. The inhibitory concentrations (IC_{50} values) were calculated using a nonlinear regression (Graphpad Prism 5.0, San Diego, CA, USA).

Results and discussion

Inhibition of a single CYP may change the safety and efficacy of a concomitant drug, especially when both drugs are metabolized by the same enzyme. For this reason, several drugs (i.e., terfenadine, cerivastatin, and nefazodone) have
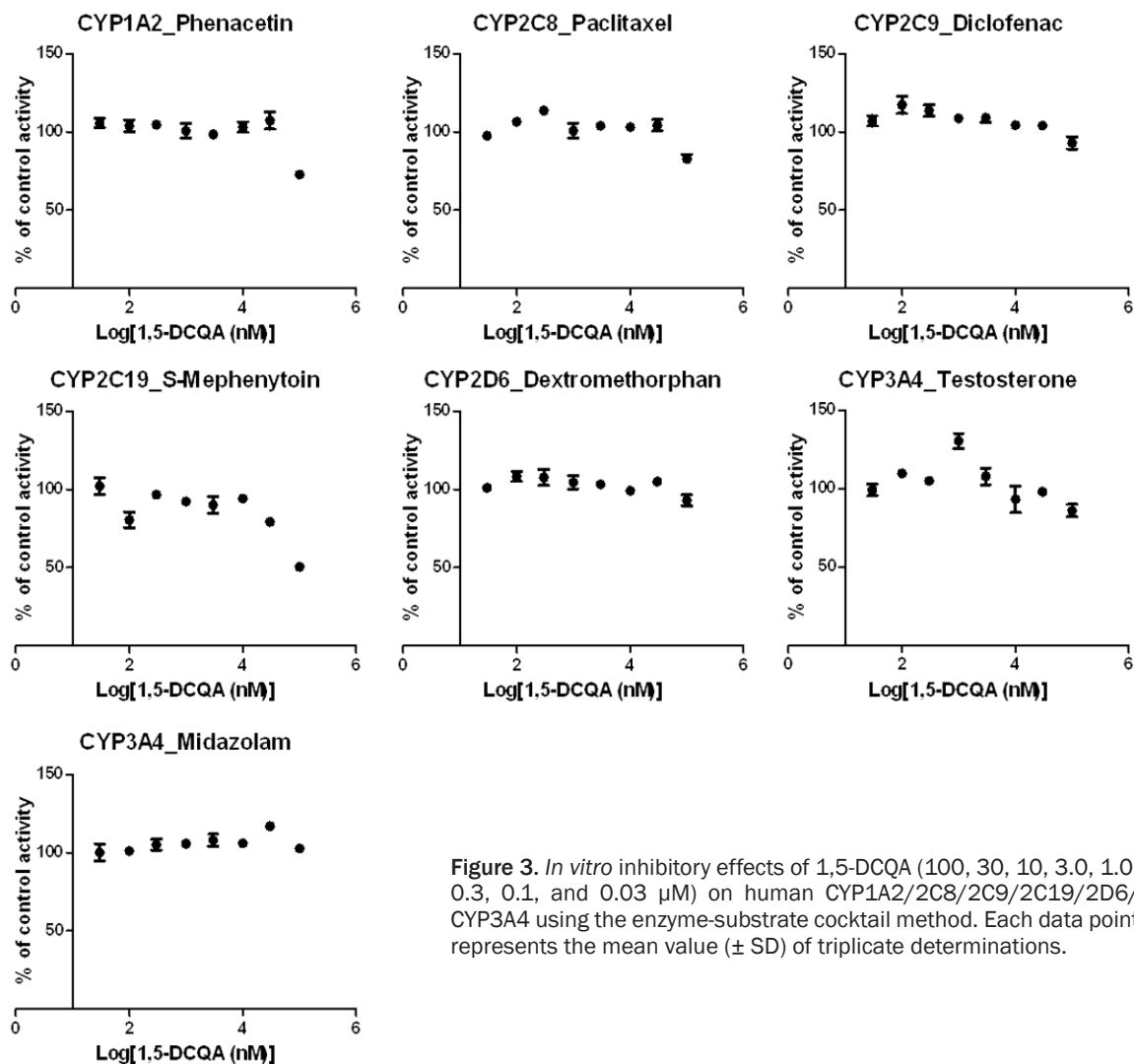
Figure 3. *In vitro* inhibitory effects of 1,5-DCQA (100, 30, 10, 3.0, 1.0, 0.3, 0.1, and 0.03 µM) on human CYP1A2/2C8/2C9/2C19/2D6/CYP3A4 using the enzyme-substrate cocktail method. Each data point represents the mean value (± SD) of triplicate determinations.

been withdrawn from the market. Thus, the FDA has suggested since 1996 that the potential for drug-drug interactions should be explored for all new drugs. Findings from *in vitro* drug interaction studies (especially using human liver microsomes) are valuable in quantitatively assessing the drug-drug interaction potential of an investigational drug. 1,5-DCQA is a promising novel drug for the treatment of HIV and HBV infection in combination with other agents. Therefore, it is important to explore the drug-drug interaction potential of 1,5-DCQA.

The chosen concentrations of each substrate, and two structurally unrelated CYP3A4 substrates used in the *in vitro* inhibition study, were in agreement with those reported by the FDA's Guidance for Industry and previous litera-

ture [37-39]. The IC_{50} values of each positive control were consistent with those reported in our published papers within an acceptable degree of accuracy [35, 36]. Therefore, it was confirmed that the study was of sufficient quality to evaluate the *in vitro* inhibitory effects of 1,5-DCQA on the six human/rat CYP isoforms.

The IC_{50} values of 1,5-DCQA on the six rat CYP isozymes (Cyp1a2, Cyp2c7, Cyp2c11, Cyp2c79, Cyp2d4, and Cyp3a2) all exceeded 100 µM (**Figure 2**). To confirm the negligible *in vitro* inhibitory effects of 1,5-DCQA on CYP isozymes, further studies demonstrated that all IC_{50} values on the six human CYP isozymes (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4) also exceeded 100 µM (**Figure 3**). These data indicated that 1,5-DCQA had negligible *in vitro* inhibitory effects on six rat and six human

major CYP isozymes. This result was consistent with that of a previous paper reporting that 1,5-DCQA had no obvious effect on CYP protein concentrations in rat liver microsomes [34]. Hence, further *in vivo* study in humans is unnecessary in accordance with the FDA's guideline.

Conclusions

In summary, 1,5-DCQA has negligible *in vitro* inhibitory effects on six rat CYPs (Cyp1a2, Cyp2c7, Cyp2c11, Cyp2c79, Cyp2d4, and Cyp3a2) and six human CYPs (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4). All IC$_{50}$ values of 1,5-DCQA are over 100 µM. These results indicate that 1,5-DCQA is unlikely to cause significant drug-drug interactions based on its effects on CYP activities.

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Disclosure of conflict of interest

None.

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