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ScienceDirect
Chinese Journal of Natural Medicines 2017, **15**(5): 0355–0362
Chinese Journal of Natural Medicines

A lipophilic prodrug of Danshensu: preparation, characterization, and *in vitro* and *in vivo* evaluation

GUO Xue-Jiao, FAN Xue-Jiao, QIAO Bin, GE Zhi-Qiang*

Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Education Ministry Key Laboratory of Systems Bioengineering, Tianjin 300072, China

Available online 20 May, 2017

[ABSTRACT] Danshensu [3-(3, 4-dihydroxyphenyl) lactic acid, DSS], one of the significant cardioprotective components, is extracted from the root of Salvia miltiorrhiza. In the present study, an ester prodrug of Danshensu (DSS), palmitoyl Danshensu (PDSS), was synthesized with the aim to improve its oral bioavailability and prolong its half-life. The *in vitro* experiments were carried out to evaluate the physicochemical properties and stability of PDSS. Although the solubility of PDSS in water was only 0.055 mg·mL^{-1}, its solubility in FaSSIF and FeSSIF reached 4.68 and 9.08 mg·mL^{-1}, respectively. Octanol-water partition coefficient (log *P*) was increased from −2.48 of DSS to 1.90 of PDSS. PDSS was relatively stable in the aqueous solution in pH range from 5.6 to 7.4. Furthermore, the pharmacokinetics in rats was evaluated after oral administration of PDSS and DSS. *AUC* and $t_{1/2}$ of PDSS were enhanced up to 9.8-fold and 2.2-fold, respectively, compared to that of DSS. C_{max} was 1.67 ± 0.11 μg·mL^{-1} for PDSS and 0.81 ± 0.06 μg·mL^{-1} for DSS. Thus, these results demonstrated that PDSS had much higher oral bioavailability and longer circulation time than its parent drug.

[KEY WORDS] Danshensu; Prodrug; Oral bioavailability; Circulation time

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2017)05-0355-08

Introduction

Danshensu (3-(3, 4-dihydroxyphenyl) lactic acid, DSS) is one of the main hydrophilic components of Radix Salvia miltiorrhiza (Danshen in China) which has been widely applied in clinical treatments in China and Japan for centuries. Its pharmacological activities have been comprehensively studied, including inhibiting platelet aggregation, decreasing the blood viscosity, scavenging oxygen free radicals, protecting the endothelial cell from homocysteine-induced dysfunction, improving heart function, and anti-inflammatory and anti-tumor activities [1-4]. However, the development of therapeutic DSS for clinical application still faces challenges because of its low oral bioavailability. DSS is administered parenterally (intravenous drip) in clinical practice due to its suboptimal biopharmaceutical properties, including the low oral bioavailability and short half-life [5]. It has been reported that the oral bioavailability of DSS in rats was only 11.09% and eliminated rapidly from the systemic circulation with $t_{1/2}$ of 45.37 min [6]. In order to increase the circulation time and improve oral bioavailability of DSS *in vivo*, several studies have been conducted. It is found that DSS phospholipid complex (DPLC) could increase the oral bioavailability of DSS since the lipophilicity of the DSS mixture was enhanced with the presence of phospholipid [7]. Also, the oral bioavailability of DSS is increased from 11.09% to 18.62% with the addition of sodium caprate, a kind of absorption enhancer [6]. These studies have revealed that the excessive aqueous solubility could lead to the inefficient absorption through the small intestinal cell membranes. Although the oral bioavailability of DSS has been improved to some extent by the aforementioned formulations, the circulation time of DSS *in vivo* are not getting better and some absorption enhancers may cause damage and irritate the intestinal mucosal membrane [8]. Hence, for the drugs with such characteristics, it is meaningful to design and synthesize the lipophilic prodrugs for altering the physicochemical properties, promoting the intestinal membrane permeation, as well as achieving the desired circulation time [9].

[Received on] 07- Dec.-2016
[Research funding] This work was supported by the National Natural Science Foundation of China (No. 31371014).
[*Corresponding author] Tel: 86-22-87401546, Fax: 86-22-27403389, E-mail: gezhiq@tju.edu.cn
These authors have no conflict of interest to declare.

The prodrug approach using reversible derivatives is useful in optimizing the clinical application of a drug [10]. Lipophilic prodrugs, also called drug-lipid conjugates, have the drug covalently bound to the lipid moiety, such as fatty acids, diglycerides, and phosphoglycerides [11]. The application of fatty acids in the formation of lipidic prodrugs has been extensively studied, such as the lipidic prodrug doxorubicin [12-13] and pomorphine [14]. As a result, it is useful to promote drug absorption and prolong the circulation time because the lipophilicity of the prodrug is enhanced dramatically, compared to the parent drug [11]. Although the lipophilic prodrug strategy by chemical modification has been widely used in the delivery of drugs, there are barely any reports on DSS prodrugs which can improve its oral bioavailability and extend its circulation half-life.

Hence, herein a palmitoyl prodrug of DSS (2-palmitoyl-3-(3, 4-dihydroxyphenyl) propanoic acid, PDSS) was designed and synthesized by introducing an alkyl-palmitoyl chain to the alcoholic hydroxyl of DSS. The palmitoylation of the drug could enhance its lipophilicity, thus improving the oral bioavailability and extending the circulation time of parent drug [15-16]. The reason that palmitic acid was used as the lipophilic moiety was its safety has been approved by the U. S. Food and Drug Administration. Moreover, the physicochemical properties, rate of hydrolysis of PDSS *in vitro*, as well as pharmacokinetics after oral administration in rats have been investigated. It was hoped that the lipophilic prodrug of Danshensu would have significant impact on its clinical application.

Materials and Methods

Chemicals and animals

DSS was purchased from Xi'an Honson Biotechnology (Xi'an, China). Palmitic anhydride (purity > 97%) and Carboxylesterase were purchased from Sigma-Aldrich (Shanghai, China). 4-Dimethylaminopyridine (DMAP, 99%), sodium borohydride ($NaBH_4$, 98%), pyridine (> 99%), and *n*-octanol (99%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. , China. Pyrene (> 97%) was purchased from Beijing Ou'he Technology Co., Ltd., China. HPLC grade acetonitrile (ACN) was bought from Merck (Darmstadt, Germany). Ultrapure water (Millipore, Schwalbach, Germany) was used as the dispersion medium. All materials used in the present study were of the highest purity available unless otherwise noted.

Synthesis of PDSS

The synthesis process of PDSS is illustrated briefly in Fig. 1. DSS (5 mmol), palmitic anhydride (16 mmol), and DMAP (0.5 mmol) were dissolved in 10 mL of anhydrous pyridine. The reaction mixture was stirred magnetically at 80 °C for 12 h. After that, the resultant reaction mixture was cooled to room temperature, to which ethyl acetate (20 mL) was added. The organic phase was washed with 5% HCL (15 mL × 2) and distilled water (15 mL × 2) successively, dried over anhydrous sodium sulfate and then evaporated at 65 °C under reduced pressure to obtain the crude product. After purification by column chromatography, the intermediate product was successfully separated.

The intermediate product (3.5 mmol) was dissolved with 10 mL of methanol, to which $NaBH_4$ (3.5 mmol) was added in batches under the magnetic stirring in ice bath. After 10 min in ice bath, the reaction temperature was raised to the reflux temperature of methanol and maintained until the end of the reaction. The target product, PDSS (2-palmitoyl-3-(3, 4-dihydroxyphenyl) propanoic acid), was obtained using the same separation and purification method as described above.

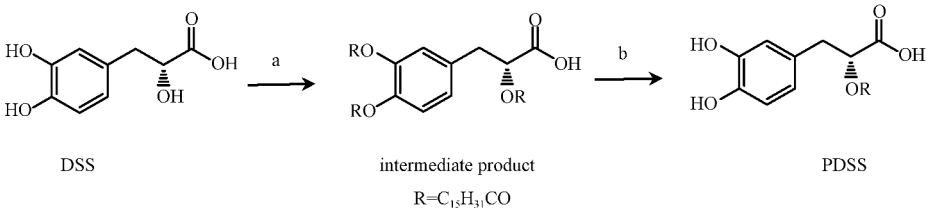

Fig. 1 Synthesis and chemical structure of PDSS. Reagents and conditions: (a) palmitic anhydride, DMAP, anhydrous pyridine, 80 °C, 12 h; (b) $NaBH_4$, methanol, reflux, 7 h

Characterization and HPLC determination of PDSS

PDSS was characterized by nuclear magnetic resonance (NMR) spectroscopy (400 MHz), high resolution mass (HR-MS) spectroscopy, and fouries transform infrared (FT-IR) spectroscopy. The purity of PDSS was determined by high performance liquid chromatography (HPLC; Waters e2695-2489uv; Waters Symetry C_{18} 5 μm, 46 mm × 250 mm column; eluent: 10% acetonitrile/90% H_2O/0.1% formic acid).

Melting point

The sample of PDSS was prepared through placing about 1 mg of PDSS on a glass slide, and the melting point was then determined by OptiMelt MPA100.

Equilibrium solubility in aqueous

Excess amounts of PDSS were added to 10 mL of distilled water in a closed Erlenmeyer flask. The oversaturated solution was placed in a shaking air bath at 25 °C and 60 $r \cdot min^{-1}$ for 24 h. After solution reaching solubility equilibrium, the precipitates were removed from the suspension through centrifugation (13 000 $r \cdot min^{-1}$ × 10 min) and the supernatant fluid was analyzed by HPLC [17].

Equilibrium solubility in FaSSIF and FeSSIF

Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF) were prepared according to the manufacturer's instructions. Ten milligram of