In Vitro Evaluation of the Effect of 7-Methyl Substitution on Glucuronidation of Daphnetin: Metabolic Stability, Isoform Selectivity, and Bioactivity Analysis

SI-CHENG LIANG,[1,2,3] GUANG-BO GE,[2] YANG-LIU XIA,[2,3] JIANG-WEI ZHANG,[2] XIAO-YI QI,[1] CAI-XIA TU,[1] LING YANG[2]

[1]Second Affiliated Hospital of Dalian Medical University, Dalian, China
[2]Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China
[3]University of Chinese Academy of Sciences, Beijing, China

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ABSTRACT: The C-8 phenol group is essential to exert the bioactivities of daphnetin, but it is readily conjugated with glucuronic acid prior to excretion. In this study, daphnetin-7-methylether (7M-DNP) was used to investigate the effect of 7-methyl substitution on daphnetin glucuronidation in human/rat liver (HLM/RLM) and intestine (HIM/RIM) microsomes, and recombinant UDP-glucuronosyltransferases (UGTs). Compared with daphnetin, the V_{max}/K_m values of 7M-DNP via 8-*O*-glucuronidation were 2.1-fold lower in HLM, 1.7-fold lower in HIM, and 2.4-fold lower in RLM, suggesting an improvement in metabolic stability. Different from daphnetin 8-*O*-glucuronidation exclusively catalyzed by UGT1A6 and UGT1A9, UGT1A1, -1A3, -1A7, -1A8, and -1A9 showed glucuronidation activity toward 7M-DNP. Kinetics studies, chemical inhibition, and the relative activity factor approach were used to demonstrate that UGT1A9 was mainly responsible for the reaction in HLM, whereas UGT1A1 was a primary contributor in HIM. The V_{max}/K_m values of 7M-DNP glucuronidation in HLM and HIM were 0.61–0.74-fold lower than those of rat, suggesting the differences between the two species. The bioactivity analysis demonstrated that 7M-DNP had an anti-inflammatory activity comparable to that of daphnetin. These findings indicated that the outcomes of 7-methyl substitution on daphnetin might be positive, but this should be confirmed in future *in vivo* studies. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3557–3564, 2015

Keywords: daphnetin; glucuronidation; methyl substitution; structural modification; metabolic stability; phase II enzymes; glucuronosyltransferases; human liver microsomes; enzyme kinetics; metabolic clearance

INTRODUCTION

An ideal drug should have a nice balance between pharmacodynamics and pharmacokinetics profiles to achieve an optimal dose-effect relationship *in vivo*.[1] Unfavorable absorption, distribution, metabolism, and excretion (ADME) properties are well-known as a major cause of failure for drug candidates.[2] To reduce high rate of failure, ADME-based structural modifications have been adopted widely as an effective approach to improve the metabolic stability of drug candidates in preclinical development.[1,2]

Daphnetin (7,8-dihydroxycoumarin), is naturally presented in plants of the genus *Daphne* and several other genera. A number of pharmacological studies show that daphnetin possesses broad spectrum of bioactivities such as anti-inflammatory, antimicrobial, and anticancer effects and protein kinase inhibitory activities.[3–7] Clinically, daphnetin not only has been approved to adjunctive therapy for cardiovascular diseases (e.g., thromboangiitis obliterans) in China since 1980s,[8] but also has been used as a folk medicine to treat lumbago and reduce fever in Turkey.[9] Additionally, owing to the positive therapeutic effects, daphnetin is recognized as the marker compound for quality control of Zushima-Pian, a traditional Chinese medicine tablet for treating rheumatoid arthritis.[10]

Despite the therapeutic benefits, daphnetin suffers from low oral bioavailability due to poor metabolic stability.[11,12] This can be ascribed, at least partly, to neglect of the ADME process of daphnetin in the early development stage. The pharmacokinetic study in rats after administration shows that daphnetin is rapidly eliminated with quite short half-live (~15 min).[11,12] The *in vitro* metabolism study with microsome incubation reveals that UGT1A6 and UGT1A9 mediated 7-*O* and 8-*O* glucuronidation are mainly responsible for daphnetin clearance in human liver and intestine.[13] The hepatic extraction ratio of daphnetin by glucuronidation is even up to 0.93, which was estimated using *in vitro* kinetic parameters obtained from HLM.[13] In addition, 7-*O* and 8-*O* glucuronides were also determined as the major metabolites of daphnetin in rat plasma.[14]

As a major phase II drug-metabolizing enzyme, UGTs are frequently involved in glucuronidation of plant-derived phenolic compounds (e.g., hydroxycoumarins and hydroxyflavones).[15,16] Currently, at least 19 human UGTs have been identified and they are classified UGT1A and UGT2A and -2B subfamilies based on amino acid sequence identity.[17] Each UGT isoform displays a tissue-specific expression pattern and in most cases, has overlapping substrates with other isoforms.[18] Normally, removing or blocking the vulnerable metabolic sites could be an effective approach to increase the metabolic

Abbreviations used: ADME, absorption, distribution, metabolism, and excretion; UGT, UDP-glucuronosyltransferase; UDPGA, uridine-diphosphate glucuronic acid; 7M-DNP, daphnetin-7-methylether; HLM, human liver microsomes; HIM, human intestine microsomes; RAF, relative activity factor; V_{max}/K_m, intrinsic clearance.

Correspondence to: Xiao-Yi Qi (Telephone: +86-411-84379317; Fax: +86-411-84676961; E-mail: 7xiao1@163.com); Ling Yang (Telephone: +86-411-84379317; Fax: +86-411-84676961; E-mail: ylingdicp@gmail.com)

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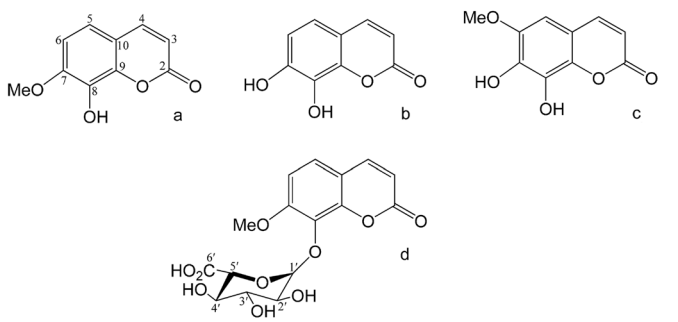

Figure 1. Chemical structures of 7M-DNP (a), daphnetin (b), fraxetin (c), and 7M-DNP glucuronide (d).

stability of drug candidates undergoing phase II reactions, including UGT-mediated glucuronidation.[1,2,19] However, when the vulnerable metabolic sites are necessary to sustain the therapeutic benefits, indirect rather than direct site modification could be a better choice.

Although both C-7 and C-8 phenols of daphnetin are vulnerable metabolic sites for glucuronidation, structure–activity studies on daphnetin and its analogues demonstrate the necessity of the C-8 phenols to their bioactivities.[7,20–23] Accordingly, the retention of C-8 phenols is crucial for designing better daphnetin derivatives as therapeutic agents. Ortho-substituent in the target phenol of chemicals is reported to be a way to enhance the metabolic stability towards glucuronidation.[24,25] For instance, because of the presence of methoxy group at C-6 position, the clearance of fraxetin (7,8-dihydroxy-6-methoxycoumarin) via 7-*O*-glucuronidation (ortho-position of C-6 atom) is largely reduced compared with daphnetin.[25] Nevertheless, fraxetin clearance via 8-*O*-glucuronidation (meta-position of C-6 atom) is increased.

In this study, we employed daphnetin-7-methylether (7M-DNP) (Fig. 1) to investigate the effect of 7-methyl substitution on daphnetin glucuronidation with human and rat tissue preparations, and recombinant UGTs. The anti-inflammatory effects of daphnetin and 7M-DNP were also determined. It is hoped that this study could provide useful information for guiding the structural modification of hydroxycoumarin derivatives with improved metabolic stability.

EXPERIMENTAL

Materials

Phenylbutazone, β-estradiol, propofol, glycyrrhetinic acid, niflumic acid, chenodeoxycholic acid, uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), β-glucuronidase (EC No. 3.2.1.31), alamethicin, Brij 58, and D-saccharic acid 1,4-lactone were purchased from Sigma–Aldrich (St. Louis, Missouri). 7M-DNP (≥98%), ethinylestradiol, and 4-methylumbelliferyl-β-D-glucuronide were purchased from Alfa Aesar (Beijing, China). Propofol glucuronide was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Cyclosporin A was purchased from J&K Chemical Ltd. (Shanghai, China). Pooled human liver microsomes (HLM) from 25 donors, and rat liver (RLM) and intestine microsomes (RIM) were purchased from Research Institute for Liver Diseases (Shanghai, China). Pooled human intestine microsomes (HIM) containing equal amounts of microsomes were prepared from both the duodenum and jejunum sections of each of the five donors (one female and four males of white and African-American race, with ages ranging from 16 to 64 years) and a panel of recombinant human UGT Supersomes (UGT1A1, -1A3, -1A4, -1A6, -1A7, -1A8, -1A9, -1A10, -2B4, -2B7, -2B15, and -2B17) expressed in baculovirus infected insect cells were purchased from BD Gentest (Woburn, Massachusetts). All other reagents were of HPLC grade or of the highest grade.

Identification of 7M-DNP Glucuronidation

The incubation mixture (200 μL) contained HLM (0.5 mg protein/mL), 5 mM UDPGA, 5 mM $MgCl_2$, 25 μg/mL alamethicin, 10 mM D-saccharic acid 1,4-lactone, 50 μM 7M-DNP and 50 mM Tris–HCl buffer (pH 7.4). After 30 min of incubation at 37°C, the reaction was terminated by the addition of 0.2 mL of acetonitrile, followed by centrifugation at 20,000×*g* for 10 min to obtain the supernatant for HPLC spectrometry analysis. Control incubations without UDPGA or without substrate or without microsomes were performed to ensure that the metabolite(s) produced was microsome and UDPGA dependent. To ensure the formation of glucuronide, the hydrolysis of the product in the incubation mixture with β-glucuronidase was also performed. After incubation for glucuronidation for 30 min in 200 μL reaction mixture (without D-saccharic acid 1,4-lactone), 200 μL 0.15 M acetate buffer (pH 5.0) with/without β-glucuronidase (1800 Fishman units) was added to the reaction mixture, and then the incubation was carried out at 37°C for another 30 min. The reaction was quenched by the addition of 200 μL acetonitrile and centrifuged at 20,000×*g* for 10 min; the supernatant was analyzed by HPLC-UV.

Analytical Instruments and Conditions

7M-DNP and its glucuronide were analyzed by an HPLC system (Shimadzu, Kyoto, Japan) containing an SCL-10A system controller, two LC-10AT pumps, a SIL-10A auto-injector, and an SPD-10AVP UV detector. The separation of 7M-DNP and its glucuronide were performed using a RP Luna (Phenomenex, Torrance, California) C_{18} column (4.6 × 150 mm^2, 5 μm). Column temperature was kept at 40°C. The mobile phase was acetonitrile (A) and water containing 0.2% formic acid (B) at a flow rate of 1 mL/min, with a gradient: 0–5 min, 95%–90% B; 5–10 min, 90%–70% B; 10–14 min, 10% B; 14–19 min, 95% B. The glucuronidation sample was stable over 72 h at 4°C. 7M-DNP glucuronidation was quantified by the standard curve of the glucuronide at the detector wavelength of 320 nm, which was linear from 0.1 to 20 μM (the correlation coefficient was 0.997). The method displayed good reproducibility, with the intra-day and inter-day variance both less than 6.3%.

Mass detection was performed on a Shimadzu LC-MS-2010EV instrument with an ESI interface both in positive and negative ion mode from *m/z* 100 to 1000. The detector voltage was set at +1.55 and −1.55 kV, for positive and negative ion detection, respectively. The curved desolations line temperature (CDL) and the block heater temperature were both set at 250°C, while the CDL voltage was set at 40 V. Other MS detection conditions were as follows: interface voltage, +4.5 and −4.0 kV for positive and negative ion detection, respectively; nebulizing gas (N_2) flow 1.5 L/min; and the drying gas (N_2) pressure 0.06 MPa. Data processing was performed using the LC–MS Solution version 3.41 software (Shimadzu).