Regio- and Isoform-Specific Glucuronidation of Psoralidin: Evaluation of 3-*O*-Glucuronidation as a Functional Marker for UGT1A9

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ABSTRACT: In this study, we aimed to determine the glucuronidation potential of psoralidin in humans and to perform validation on use of psoralidin-3-*O*-glucuronidation as a functional marker for UGT1A9. Glucuronidation kinetics was determined using human liver microsomes (HLMs), human intestine microsomes (HIM), and expressed UDP-glucuronosyltransferase (UGT) enzymes. The chemical structures of metabolites were determined by liquid chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy analyses. Validation of psoralidin-3-*O*-glucuronidation as a UGT1A9 marker was performed using combined approaches including reaction phenotyping, chemical inhibition, activity correlation analysis, and determination of relative activity factor (RAF). HLM and UGT1A9 generated two monoglucuronides (9-*O*-glucuronide and 3-*O*-glucuronide) from psoralidin, whereas HIM, UGT1A1, UGT1A7, and UGT1A8 generated one only (9-*O*-glucuronide). Formation of 3-*O*-glucuronide in HLM was markedly inhibited by the UGT1A9-selective inhibitors magnolol and niflumic acid. Further, psoralidin-3-*O*-glucuronidation was strongly correlated with propofol–glucuronidation in a group of nine individual HLMs ($r = 0.978$, $p < 0.001$). Strong correlation was also observed between psoralidin-3-*O*-glucuronidation and the UGT1A9 protein levels measured by Western blotting ($r = 0.944$, $p < 0.001$). Moreover, UGT1A9 was responsible for 99.6% of psoralidin-3-*O*-glucuronidation in HLM based on the RAF approach. In conclusion, psoralidin was subjected to efficient glucuronidation, generating one or two monoglucuronides depending on UGT isozymes. Also, psoralidin-3-*O*-glucuronidation was an excellent *in vitro* marker for UGT1A9. J Pharm Sci 104:2369–2377, 2015
Keywords: glucuronidation; UGTs; psoralidin; functional marker; reaction phenotyping

INTRODUCTION

Drug disposition in the body is rather complex, involving absorption, distribution, metabolism, and excretion processes (or ADME). Poor ADME properties are associated with a high rate of drug attrition.[1,2] Hence, ADME studies, as an important factor to decision making, have been closely integrated into the drug discovery and development programs.[3] Metabolism is a critical ADME property that determines the bioavailability and efficacy/toxicity of drugs. Drug metabolism reactions can be categorized into phase I and phase II reactions. Phase I reactions include the oxidation, reduction, and hydrolysis reactions.[4] Phase II reactions refer to various types of conjugation reactions such as glucuronidation and sulfation.[5] This "phase" classification system is based on early observations that phase II enzymes tend to conjugate drugs that have undergone phase I metabolism. However, it is well accepted nowadays that in addition to conjugating phase I metabolites, phase II enzymes can also directly metabolize many drugs (e.g., raloxifene and gemfibrozil).[6]

UDP-glucuronosyltransferase (UGT) enzymes are a family of glycotransferases that mediate the glucuronidation reaction, an important metabolic and detoxification pathway for numerous endogenous compounds (e.g., bilirubin and estradiol) and xenobiotics (e.g., dietary polyphenols and drugs).[7] In glucuronidation reactions, the glucuronic acid derived from the cofactor UDPGA (uridine diphosphoglucuronic acid) is transferred to the substrates, producing the glucuronidated metabolite or glucuronide.[8] Compared with the parent compound, the generated glucuronide are much more readily eliminated from the body because of increased water solubility. Human UGTs constitute a number of enzymes that are divided into five families, namely, UGT1A, UGT2A, UGT2B, UGT3A, and UGT8A.[9,10] The UGT1A and 2B enzymes appear to be the main contributors to glucuronidation of xenobiotics including drugs.[10] UGT1A family consists of nine members (i.e., UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10), whereas UGT2B family consist of seven members (i.e., UGT2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28).[9] Body distribution of UGT enzymes at the mRNA level has been well established.[11–13] More recently, protein levels of UGTs in orangs of metabolism were assessed using targeted quantitative proteomics with liquid chromatography–mass spectrometry (MS).[14–16] Human liver abundantly expresses UGT1A1, 1A9, 2B7, and 2B15.[14–16] UGT1A1 and 2B17 are significantly expressed in the intestine, whereas UGT1A9 and 2B7 are found in the kidney.[15]

Psoralidin (3,9-dihydroxy-2-(3-methylbut-2-enyl)-[1]benzofuro[3,2-c]chromen-6-one) is a natural coumestan isolated from the seeds of *Psoralea corylifolia*, a medicinal

Abbreviations used: K_m, Michaelis–Menten constant; MS, mass spectroscopy; NMR, nuclear magnetic resonance spectroscopy; UDPGA, uridine diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC, ultra-performance liquid chromatography.

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plant used in Asia for management of various diseases such as cardiovascular diseases, inflammatory diseases, hypertension, and nephritis diseases.[17,18] It has been demonstrated that psoralidin processes a variety of biological activities such as antibacterial,[19] antioxidant,[20] anti-inflammatory,[21] and antidepressant effects.[22] In recent years, numerous studies indicate that psoralidin is a potential anticancer agent.[23–26] Despite that psoralidin showed many types of health benefits, very little is known about its metabolic profiles.

UDP-glucuronosyltransferase enzymes showed extensive overlaps in substrate selectivity, posing significant difficulties in identification of probe substrates for a specific isozyme.[27] To date, a limited number of probe substrates are available for UGT enzymes.[28] The lack of *in vivo* probe substrates appears to be one main obstacle to studying UGT functions and determining the exact contributions of UGTs to drug metabolism.[29] UGT1A9 is an important UGT member, showing board substrate selectivity.[30] Propofol is the only probe substrate of UGT1A9 that has gained wide acceptance for uses in *in vitro* glucuronidation studies.[28] Clearly, finding probe substrates (particularly those show great potential in *in vivo* applications) for UGT1A9 is still a major task in the field.

Because of the great potential in treating many types of diseases, it is of great value to determine the metabolic pathways of psoralidin in humans. The objective of the present study was to characterize metabolism of psoralidin via the glucuronidation pathway. To this end, glucuronidation kinetics of psoralidin was determined using human liver microsomes (HLMs), human intestine microsomes (HIMs), and expressed UGT enzymes. The chemical structures of generated glucuronides were determined by high-resolution masses and nuclear magnetic resonance spectroscopy (NMR) analyses. Further, the use of psoralidin 3-*O*-glucuronidation as a functional marker for UGT1A9 was proposed and validated by using combined approaches including reaction phenotyping, chemical inhibition, activity correlation analysis, and determination of relative activity factor (RAF). We demonstrated for the first time that psoralidin was subjected to efficient glucuronidation, generating one or two monoglucuronides depending on the exact UGT isozymes. We also provided strong evidence that psoralidin-3-*O*-glucuronidation was an excellent *in vitro* marker for UGT1A9.

MATERIALS AND METHODS

Materials

Pooled HLMs, pooled HIMs, and expressed UGT enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) were purchased from BD Biosciences (Woburn, Massachusetts). UDPGA, alamethicin, D-saccharic-1,4-lactone monohydrate, β-glucuronidase (from *E. coli*), and propofol were purchased from Sigma–Aldrich (St Louis, Missouri). Psoralidin was purchased from Aladdin Chemicals (Shanghai, China). Magnolol and niflumic acid were purchased from Nanjing Zelang Medicine Technology Company Ltd. (Nanjing, China). The anti-UGT1A9 antibody was purchased from Abcam (Cambridge, Massachusetts). Individual HLMs ($n = 9$) obtained from healthy livers were purchased from Rild Research Institute for Liver Diseases (Shanghai, China). All other materials (typically analytical grade or better) were used as received.

Glucuronidation Assay

The glucuronidation assay was performed as described in our previous publications.[31–34] The stock solutions of aglycones were prepared in methanol–dimethyl sulfoxide (DMSO) (4:1, v/v) and diluted with methanol–DMSO (4:1, v/v) to the desired concentrations immediately before use. Incubation mixtures contained HLM/HIM or expressed UGTs (13.3 μg/mL), $MgCl_2$ (0.88 mM), saccharolactone (4.4 mM), alamethicin (22 μg/mL), and aglycones (at desired concentrations) in 50 mM potassium phosphate (pH 7.4). Final incubation volumes were 300 μL and the total organic solvent content was 1%. The reactions were initiated by addition of UDPGA (3.5 mM) at 37°C for 30 min in a water bath and terminated by adding 100 μL ice-cold acetonitrile. Samples were centrifuged at 18,000*g* for 15 min, and the supernatant was analyzed by ultra-performance liquid chromatography (UPLC). Preliminary experiments were performed to ensure that the rates of glucuronidation were determined under linear conditions with respect to the incubation time and protein concentration. All experiments were performed in triplicate.

Kinetic Evaluation

The rates of glucuronidation were determined for psoralidin at a series of concentrations (i.e., 0.625–10, 0.625–10, 0.313–10, 0.625–10, 1.88–20, and 0.625–10 μM for HLM, HIM, UGT1A1, UGT1A7, UGT1A8, and UGT1A9, respectively) according to the glucuronidation assay protocol. The kinetic model Michaelis–Menten (Eq. (1)) or Hill equation (Eq. (2)) was fitted to the data of glucuronidation rates versus substrate concentrations. Model selection was based on visual inspection of the Eadie–Hofstee plot.[35,36] Model fitting and parameter estimation were performed using the Graphpad Prism V5 software (San Diego, California).

$$V = \frac{V_{\max}[S]}{K_m + [S]} \tag{1}$$

$$V = \frac{V_{\max}[S]}{K_m + [S]\left(1 + \frac{[S]}{K_{si}}\right)} \tag{2}$$

where V_{max} is the maximal velocity, K_m is the Michaelis–Menten constant, and K_{si} is the substrate inhibition constant. The intrinsic clearance (CL_{int}) was derived by V_{max}/K_m.

Biosynthesis of Psoralidin Glucuronides

A large amount of psoralidin glucuronides were generated using HLMs according to the glucuronidation assay protocol. The glucuronides were isolated and purified as described.[33] In brief, the incubation mixture after reaction was concentrated and injected into the Dionex U300 HPLC system. Psoralidin and its glucuronides (G1 and G2) were separated by the Agilent TC-C18(2) column (5 μm, 4.6 × 250 mm) using a gradient method with acetonitrile (B)–water(A) as the mobile phases (10%–35% B at 0–15 min, 35%–80% B at 15–20 min, 80% B at 20–22 min, and 80%–10% B at 22–24 min) at a flow rate of 1 mL/min. Fractions containing G1 or G2 were collected respectively and dried *in vacuo*.