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Regular Article

In vitro inhibitory effects of major bioactive constituents of *Andrographis paniculata*, *Curcuma longa* and *Silybum marianum* on human liver microsomal morphine glucuronidation: A prediction of potential herb-drug interactions arising from andrographolide, curcumin and silybin inhibition in humans

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ABSTRACT

This study aimed to investigate the liver microsomal inhibitory effects of silybin, silychristin, andrographolide, and curcumin by using morphine as an *in vitro* UGT2B7 probe substrate, and predict the magnitude of the herb-drug interaction arising from these herbal constituents' inhibition *in vivo*. Studies were performed in the incubation with and without bovine serum albumin (BSA). Andrographolide and curcumin showed a marked inhibition on morphine 3- and 6-glucuronidation with IC_{50} of 50&87 and 96&111 μM, respectively. In the presence of 2%BSA, andrographolide also showed a strong inhibition on morphine 3- and 6-glucuronidation (IC_{50} 4.4&21.6 μM) whereas curcumin showed moderate inhibition (IC_{50} 338&333 μM). In the absence and presence of 2%BSA, morphine 3- and 6-glucuronidation was moderately inhibited by silybin (IC_{50} 583&862 and 1252&1421 μM, respectively), however was weakly inhibited by silychristin (IC_{50} 3527&3504 and 1124&1530 μM, respectively). The K_i of andrographolide, curcumin and silybin on morphine 3- and 6-glucuronidation were 7.1&9.5, 72.7&65.2, and 224.5&159.7 μM, respectively, while the respective values generated from the system containing 2%BSA were 2.4&3.1, 96.4&108.8, and 366.3&394.5 μM. Using the *in vitro* and *in vivo* extrapolation approach, andrographolide was herbal component that may have had a potential interaction *in vivo* when it was co-administered with morphine.

1. Introduction

Morphine is an opioid that is used widely in clinical practice for its analgesic properties. The major metabolic pathway in the liver with respect to morphine involves glucuronidation via the hydroxyl group at the 3- and 6- positions, yielding water-soluble compounds [1]. Elimination by 3- and 6-glucuronidation represents 57% and 10% of the total morphine systemic clearance, respectively [1]. Glucuronidation is a synthetic reaction in phase II drug biotransformation that involves the conjugation of glucuronic acid, derived from the cofactor UDP-glucuronic acid, to a substrate bearing a suitable functional group [2]. This reaction is catalyzed by enzymes known as the UDP-glucuronosyltransferases (UGTs). UGTs exist as an enzyme superfamily, and nineteen human UGT enzymes that use UDP-glucuronic acid as a co-factor have been identified to date [2]. Of these, UGT 1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 are known as the enzymes that play a major role in hepatic drug and xenobiotic metabolism [2]. However, UGT2B7 is the major enzyme responsible for hepatic morphine 3- and 6-glucuronidation [3–5].

The use of herbal supplements has risen over the last decade. Several surveys in the United States found that approximately 20% of patients took prescribed medications concomitantly with herbal medicines [6,7], leading to the potential for herb-drug interactions. The effect of herbs on the cytochrome P450 enzyme has been extensively reported [8,9]. Some of the interactions can lead to treatment failure and toxicity; for example, St. John's wort leads to graft rejection in patients receiving cyclosporine [10], and chokeberry extract may potentiate trabectedin-induced

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rhabdomyolysis [11]. However, the effects of herbal supplements on the glucuronidation pathway with respect to drugs have been inadequately studied.

Milk thistle (*Silybum marianum*) is the top-selling herbal supplement and is commonly used for its hepatoprotective [12,13] and anticancer effects [14,15]. A survey also reported that milk thistle is the fourth most frequently used herb in cancer patients [16]. Silymarin is a mixture extract of polyphenolic flavonoids from milk thistle and is composed of flavonolignans, including silybin A and B (48–57%), silychristin (22–24%), silydianin (9–15%), and isosilybinin A and B (11–13%) [17]. Glucuronidation has been identified as the major metabolic pathway of these flavonolignans in humans. Wen et al. [17] found that the fractions of free, sulfated and glucuronidated silymarin in human plasma after administration of milk thistle extract were approximately 17, 28 and 55% of the total, respectively. Silibinin monoglucuronide and diglucuronide have also been identified as the major metabolites in colorectal cancer patients who receive silybin [18]. In addition, several *in vitro* experiments have shown the inhibitory effect of silymarin flavonolignans on UGT activities [19]. Using 7-hydroxy-4-(trifluoromethyl) coumarin as a substrate, silybin can inhibit UGT 1A1, 1A6, 1A9, 2B7 and 2B15 with IC_{50} of 1.4, 28, 20, 92 and 75 μM, respectively [20].

Andrographolide and curcumin, which are the leading herbal medicines used in Asia, are the major bioactive components of *Andrographis paniculata* and *Curcuma longa*, respectively. Andrographolide has a wide range of biological effects including anti-inflammatory, anti-allergic, antiviral and immune-stimulator properties [21,22], while curcumin has antineoplastic and anti-inflammatory effects [23]. In Thailand, *Andrographis paniculata* and *Curcuma longa* have been promoted by the healthcare system as herbal supplements and were included in The National List of Essential Herbal Drugs. Several *in vitro* studies have shown that UGT2B7 involves hepatic glucuronidation of andrographolide and curcumin in humans [24,25], and both compounds have shown a potent inhibition of UGT2B7 activities [26,27]. Ismail et al. found that *Andrographis paniculata* extract can inhibit UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A10 and 2B7, with IC_{50} values of 5.0, 1.7, 5.7, 9.9, 2.6, 15 and 2.8 μg/ml, respectively [26]. In addition, Taesotikul et al. [27] found that the ethanolic extracts of *Andrographis paniculata* and *Curcuma longa* were potent inhibitors of UGT2B7, with IC_{50} values of 12.5 ± 7.1 and 11.2 ± 4.3 μg/ml, respectively.

The development and use of *in vitro* approaches to predict drug clearance and drug interactions *in vivo* (*in vitro* and *in vivo* extrapolation; IVIVE) has found increasing acceptance, not only to assist in the selection of new drug candidates but also for the rationalization and optimization of dosage regimens for established drugs. There are studies illustrating that kinetic parameters generated *in vitro* by using human liver microsomes (HLMs) and/or recombinant human UGTs as enzyme sources can predict the magnitude of *in vivo* inhibitory interactions involving glucuronidated drugs. In particular, the predictions were successful for UGT2B7 substrates performing in the *in vitro system* containing bovine serum albumin such as morphine [28], codeine [29], zidovudine [30] and lamotrigine [31]. This study aims to investigate the inhibitory effect of the major bioactive constituents of *Silybum marianum*, *Andrographis paniculata* and *Curcuma longa* (viz; silybin and silychristin, andrographolide, and curcumin, respectively; See chemical structure on Supplement) on UGT2B7 activities by using morphine as the *in vitro* probe substrate. In addition, based on the reported human pharmacokinetic data of these herbal constituents, the magnitude of herb-drug interactions arising from inhibition of morphine 3- and 6-glucuronidation by these compounds *in vivo* will be predicted using the IVIVE approach.

2. Materials and methods

2.1. Chemicals and reagents

Alamethicin (from *Trichoderma viride*), andrographolide, bovine serum albumin (BSA), curcumin, pooled human liver microsomes (pooled HLMs; M0317), silybin, silychristin and UDP-glucuronic acid (UDPGA; trisodium salt) were purchased from Sigma-Aldrich (Singapore); morphine sulfate from Department of medical sciences, Ministry of public health (Bangkok, Thailand); morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G) from Lipomed AG (Arlesheim, Switzerland); and the rapid equilibrium dialysis (RED) device single-use plate with inserts, 8.0 kDa molecular weight cut-off from Thermo Scientific (Rockford, IL, USA). Solvents and other reagents were of analytical reagent grade.

2.2. Morphine 3- and 6- glucuronidation kinetic by pooled human liver microsomes

Morphine 3- and 6- glucuronide (M3G and M6G) formation by pooled human liver microsomes (HLMs) in the absence and presence of 2% (w/v) bovine serum albumin (2% BSA) were measured by using method which modified from previous studies [28,32]. In brief, the incubation mixture (in a total volume of 200 μl) contained phosphate buffer (0.1 M, pH 7.4), $MgCl_2$ (4 mM), alamethicin-activated pooled HLMs (0.3 mg/ml), and morphine. Morphine was used in the concentration range of 0.1–15 mM and 0.025–10 mM for the reaction performed in the absence and presence of 2% BSA, respectively. Reactions were initiated by the addition of UDPGA (5 mM) and incubated at 37 °C in a shaking water bath for 60 min. The rate of M3G and M6G formation at morphine concentration of 0.1 mM was optimized for linearity with respect to incubation time. Reactions in the absence and presence of 2% BSA were terminated by the addition of 2 μl and 8 μl of 70% (v/v) $HClO_4$, respectively. Samples were centrifuged (5000*g*) at 10 °C for 10 min, and a 20 μl aliquot of the supernatant fraction was injected into the HPLC column.

2.3. Quantification of morphine 3- and 6-glucuronide formation by using HPLC

HPLC condition was modified from previous studies [28,32]. HPLC was performed using an Agilent 1100 series instrument (Agilent Technologies, Bangkok, Thailand) fitted with a NovaPak C18 column (3.9 × 150 mm, 4 μm particle size; Waters, Milford, MA, USA). The column eluent was detected by using the fluorescence at excitation and emission wavelengths of 235 nm and 345 nm, respectively. The mobile phase consisted of 1-octanesulfonic acid (4 mM), acetonitrile (10%) and glacial acetic acid (1%) in distilled water, and adjusted to pH 2.6. It was isocratically run at flow rate of 1 ml/min. Retention times for M3G, M6G and morphine were 6.0, 11.6 and 22.0 min, respectively. To quantify M3G and M6G concentrations in the incubation, their peak areas were compared with those of standard curves prepared over the concentration ranges 2.5–20 and 0.5–4 μM, respectively. Overall within-day reproducibility of M3G and M6G formation in 6 separate incubations without 2%BSA at morphine concentration of 0.25 and 10 mM were less than 10%.

2.4. Inhibition of human liver microsomal morphine glucuronidation by herbal constituents

Accumulating evidences have shown that the addition of BSA for incubation can sequester the membrane long-chain unsaturated fatty acids that are released from enzyme sources including

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