Journal of Ethnopharmacology 163 (2015) 149–156

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

An ex vivo approach to botanical–drug interactions: A proof of concept study

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ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form
12 January 2015
Accepted 15 January 2015
Available online 24 January 2015

Keywords:
Botanical–drug interactions
Cytochrome P450s
Ex-vivo model
Milk thistle
Goldenseal

ABSTRACT

Ethnopharmacological relevance: Botanical medicines are frequently used in combination with therapeutic drugs, imposing a risk for harmful botanical–drug interactions (BDIs). Among the existing BDI evaluation methods, clinical studies are the most desirable, but due to their expense and protracted time-line for completion, conventional in vitro methodologies remain the most frequently used BDI assessment tools. However, many predictions generated from in vitro studies are inconsistent with clinical findings. Accordingly, the present study aimed to develop a novel ex vivo approach for BDI assessment and expand the safety evaluation methodology in applied ethnopharmacological research.

Materials and methods: This approach differs from conventional in vitro methods in that rather than botanical extracts or individual phytochemicals being prepared in artificial buffers, human plasma/serum collected from a limited number of subjects administered botanical supplements was utilized to assess BDIs. To validate the methodology, human plasma/serum samples collected from healthy subjects administered either milk thistle or goldenseal extracts were utilized in incubation studies to determine their potential inhibitory effects on CYP2C9 and CYP3A4/5, respectively. Silybin A and B, two principal milk thistle phytochemicals, and hydrastine and berberine, the purported active constituents in goldenseal, were evaluated in both phosphate buffer and human plasma based in vitro incubation systems.

Results: Ex vivo study results were consistent with formal clinical study findings for the effect of milk thistle on the disposition of tolbutamide, a CYP2C9 substrate, and for goldenseal's influence on the pharmacokinetics of midazolam, a widely accepted CYP3A4/5 substrate. Compared to conventional in vitro BDI methodologies of assessment, the introduction of human plasma into the in vitro study model changed the observed inhibitory effect of silybin A, silybin B and hydrastine and berberine on CYP2C9 and CYP3A4/5, respectively, results which more closely mirrored those generated in clinical study.

Conclusions: Data from conventional buffer-based in vitro studies were less predictive than the ex vivo assessments. Thus, this novel ex vivo approach may be more effective at predicting clinically relevant BDIs than conventional in vitro methods.

1. Introduction

Botanical supplements typically contain numerous constituents and are extensively promoted for their putative health benefits. Their use has grown steadily over the last 10–15 years (Mukherjee et al., 2011), which has always played a vital role in applied ethnopharmacological studies (Heinrich et al., 2009). Surveys reveal that those suffering with chronic disease states are more likely to combine conventional medications with botanical products, thereby increasing the risk for botanical–drug interactions (BDI) (Choi et al., 2011).

There have been numerous in vitro assessments of BDIs using various assays including microsomal systems, "supersomes", cytosols, expressed enzymes, cell cultures, and others (Agarwal et al., 2014). Less commonly, in vivo studies have been used to characterize BDIs. The time, expense, and safety concerns associated with in vivo studies often make them prohibitive to perform (Gurley et al., 2012). Animal models, particularly rodents, though relatively inexpensive, have a number of significant translational limitations (Goey et al., 2014). Thus, in vitro methods continue to be the most widely utilized means

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http://dx.doi.org/10.1016/j.jep.2015.01.021
0378-8741/

for assessing CYP-mediated inhibitory BDIs. However, relatively few "positive" in vitro BDI predictions have been confirmed clinically (Markowitz and Zhu, 2012).

Milk thistle (*Silybum marianum* [MT]), a popular botanical product purported to convey hepatoprotection, provides an excellent example of the conflicting results noted between in vitro predictions and in vivo realities. The purported active phytochemicals of MT consist of seven flavonolignans, collectively termed silymarin, of which the most abundant are the silybinin diastereoisomers: silybin A and silybin B (Zhu et al., 2013). Potentially significant inhibition of CYP2C9, 3A and major hepatic UDP-glucuronosyltransferases (UGTs) by silymarin components have been reported in several in vitro studies (Beckmann-Knopp et al., 2000; Brantley et al., 2010; Doehmer et al., 2011; Sridar et al., 2004). However, most clinical BDI investigations have failed to confirm any clinically relevant BDI (Gurley et al., 2004; Kawaguchi-Suzuki et al., 2014; Rajnarayana et al., 2004).

Conversely, there are some examples of clinical studies confirming effects predicted by in vitro studies. One such example is goldenseal (*Hydrastis canadensis* [GS]), a botanical purported to be useful in the treatment of gastrointestinal ailments, colds symptoms, etc (Junio et al., 2011). Goldenseal extracts contain an array of phytoconstituents – ~28 plant alkaloids have been identified to date (Le et al., 2013). However, hydrastine (consisting of (-)-β-hydrastine and (-)-α-hydrastine) and berberine are generally believed to be the two principal bioactive components (Abourashed and Khan, 2001). Several in vitro studies have demonstrated that both GS extracts and individual alkaloids can inhibit CYP2C9, 2D6, and 3A4 activity (Chatterjee and Franklin, 2003; Etheridge et al., 2007) and a significant inhibitory effect of GS on CYP2D6 and CYP 3A4/5 activity has been confirmed by clinical studies (Gurley et al., 2005; Gurley et al., 2008).

There are a number of shortcomings of in vitro study methodology directed at BDIs. These include difficulty in assigning physiologically relevant hepatic drug/phytochemical concentrations; accounting for first pass metabolism and resulting metabolites; and an absence of endogenous proteins, hormones, metabolites, etc., which may exert uncertain influences, from typical buffer solutions (Wienkers and Heath, 2005). Standard in vitro CYP assays are performed under artificial conditions. Accordingly, factors such as buffer strength and pH, the presence of divalent cations, and organic solvents can potentially confound the results of these assays (Ong et al., 2013). Assessments of BDIs pose further unique challenges beyond those of conventional medications including limited availability of phytochemical reference standards (especially metabolites), absent or limited human pharmacokinetic data describing bioavailability or metabolism, inability to accurately screen botanical mixtures, and limited knowledge of solubility in physiologic solutions. These obstacles have likely contributed to the discrepancies between BDI predictions generated by in vitro methods and those observed in vivo (Markowitz et al., 2008). The aim of the present study was to develop an ex vivo model which combined the advantages of both in vitro and in vivo methods so as to assess BDIs more quickly, less expensively, and in greater agreement with clinical assessments.

We hypothesized that incubating enzymes with human plasma or serum from research subjects who had ingested specific dietary supplements in a controlled environment would provide a more clinically relevant representation of multi-constituent botanical products compared to standard in vitro approaches. Additionally, this approach would permit the assessment of parent constituents and metabolites (known and unknown) in their proportions as occur in the systemic circulation. Moreover, the effects of plasma/serum protein binding to these constituents would provide a better representation of the in vivo milieu. To test our hypothesis, we developed an ex vivo model and validated it by assessing the effects of MT and GS extracts on the activity of CYP2C9 and 3A, respectively.

2. Materials and methods

2.1. Chemicals and reagents

Silybin A, silybin B, hydrastine, berberine were from Phytolab GmbH & Co. (Vestenbergsgreuth, Germany), tolbutamide (TOLB), 4-hydroxy-tolbutamide (4-hydroxy-TOLB), d9-tolbutamide (d9-TOLB) (IS) were obtained from TLC PharmaChem (Ontario, Canada). Midazolam (MDZ) and 1′-hydroxy-midazolam (1′-hydroxy-MDZ) were the products from Cerilliant Corporation (Round Rock, TX). Phenacetin (IS) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes were obtained from BD Biosciences (Woburn, MA). Blank human plasma was sourced from a local blood bank (J.S.M.) and was pooled from 5 different donors. All other chemicals and reagents were of the highest analytical grade commercially available.

2.2. Clinical plasma specimens containing phytoconstituents

The clinical samples utilized in the present study were sourced from banked residual blood samples stored at −70 °C from completed normal volunteer pharmacokinetic studies of MT extracts (J.S.M.) and GS extracts (B.J.G.) in which subjects had consented to the use of any of their leftover samples for further research purposes. The MT specimens consisted of multiple plasma samples and were collected at the Medical University of South Carolina (MUSC) General Clinical Research Center (GCRC) in Charleston, SC where each subject provided written informed consent approved by the MUSC Office of Research Integrity. The GS specimens were collected as multiple serum samples and were collected at the University of Arkansas for Medical Sciences (UAMS) Clinical Research Services Core in Little Rock, AR where each subject provided written informed consent approved by the UAMS Human Research Advisory Committee. Five Caucasian subjects from each respective study group were utilized in the BDI assessments. The volunteers from the milk thistle and goldenseal pharmacokinetic studies consisted of 3 males, 2 females (26.3 ± 5.7 years, 53.7 ± 6.8 kg) and 4 males, 1 female (31.0 ± 4.9 years, 75.4 ± 16.2 kg), respectively. Both plasma and serum samples utilized in the present investigation were obtained from study participants who were nonsmokers, not taking prescription or over-the-counter medications, and who were not taking botanical or dietary supplements (inclusive of vitamins) as requirements of their respective study protocols. Further, the participants were also requested to abstain from grapefruit juice and caffeine-containing beverages, and ethanol 2 weeks prior to and during active study periods.

2.3. Conventional in vitro assessment of CYP2C9 inhibition by silybin A and silybin B as well as CYP3A inhibition by hydrastine and berberine

Silybin A, silybin B and hydrastine, berberine were selected to represent the principal components of MT and GS, respectively. Pooled human liver microsomes were pre-incubated with an NADPH generating system (0.1 mg/ml yeast glucose-6-phosphate dehydrogenase, 3 mg/ml NADP+, and 0.07 M glucose-6-phosphate), in the presence and absence of various concentrations of silybin A (1, 3, 10, 30, 100 μM), silybin B (1, 3, 10, 30, 100 μM), hydrastine (3, 10, 30, 100, 300 μM), and berberine (30, 100, 300, 600, 1000 μM) in 0.1 mM phosphate buffer at 37 °C for 10 min. The reactions for the inhibitory effect assessment of MT and GS were initiated by adding the probe substrates of CYP2C9 (TOLB) and CYP3A4 (MDZ) to a final volume of 80 μl, respectively. The final concentrations of the human liver microsomes were 1 mg/ml for TOLB and 0.2 mg/ml for MDZ, and the final substrate concentrations of TOLB and MDZ were 400 μM and