Mol. Nutr. Food Res. 2008, *52*, 747 – 754 DOI 10.1002/mnfr.200700159

Review

Predicting interactions between conventional medications and botanical products on the basis of *in vitro* investigations

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The potential for various natural products to perturb the metabolism and disposition of medications has been recognized for decades. There are numerous *in vitro* and *in vivo* methods available to screen botanical products for drug interaction potential. Although many normal volunteer botanical-drug interaction studies have been performed, clearly, *in vitro* studies assessing the potential for drug interactions with various natural products represent the predominant type of published research performed to date. In addition to the recognized limitations of *in vitro* screening methodologies to assess conventional drug interactions, further difficulties emerge when examining botanical products. Primary challenges include assigning hepatic concentrations and accounting for bioavailability, distribution, first-pass metabolism and active metabolites. Additionally, variability in the chemical composition of commercially available botanical supplements, the lack of analytical standards and the inability to accurately screen the entities as mixtures add to complexities in experimental design. This mini-review is intended to address the particular problems and challenges in evaluating botanical supplements using *in vitro* methods, and review what can and cannot be learned from such investigations.

Keywords: Botanical / Interaction / *In vitro* / Metabolism

Received: April 26, 2007; revised: July 24, 2007; accepted: August 8, 2007

1 Introduction

Products containing biologically active phytochemicals are widely available for use in the US. They are often termed "herbal" or "botanical" supplements and are derived from a wide variety of plant sources. They can be purchased in numerous forms the most common of which are encapsulated extracts. Extracts containing natural phytochemicals are also available as beverages and tinctures and can be ingredients in sports/nutrition drinks, powders, and "energy" bars. Food products containing specific phytochemical constituents are also being used by the public for the treatment of some common ailments (*e.g.* cranberry juice for urinary tract infections, garlic for hyperlipidemia). Natural products do not have to be consumed as "medicines" to be involved in a drug interaction. A notable example is grapefruit juice, components of which have been demonstrated in both *in vitro* and numerous clinical studies to increase the bioavailability of numerous cytochrome P450 (CYP) 3A substrates [1, 2]. Another example is cruciferous vegetables which have been implicated in interacting with a number of CYP1A2 substrates [3].

The use of supplements containing phytochemicals in the US has grown at an unprecedented rate over the past decade following passage of the Dietary Supplement and Health Education Act of 1994. For example, in 1995 and 1996, >20% increases in botanical supplement sales occurred over the respective preceding years [4]. After a short period of decline in sales, it appears that herbal supplement sales have increased in the years 2004 and 2005 [5]. According to the US Food and Drug Administration, an astounding 29000 dietary supplement products may be available to consumers [6]. Additionally, the marketing of botanical combination supplements adds further layers of

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Abbreviations: **CYP**, cytochrome P450; **P-gp**, P-glycoprotein; **SJW**, St. John's wort; **UGT** uridine diphosphoglucuronosyltransferase

complexity to the assessment of potential for drug interactions. According to the National Center for Complementary and Alternative Medicine website, 19% of Americans reported using a natural or botanical product for a specific purpose related to complementary and alternative medicine (http://nccam.nih.gov/news/report.pdf). The survey also revealed that most people use the natural or botanical products concomitantly with conventional medicine rather than in place of conventional medicine (*i.e.* complementary rather than alternative).

The potential influence of various natural products upon the metabolism and disposition of medications has been recognized for decades, well before the recent and drastic increase in use of these agents by the general public. The discovery that one of the most popular dietary supplements, St John's wort (SJW), and essentially, the first dietary supplement implicated in significant and unequivocal botanical-drug interactions [7] served notice that this example could well represent but the "tip of the iceberg" of literally thousands of available botanical supplements many of which could likewise interact significantly with concomitant medications. Consequently, efforts to screen numerous botanical products were soon underway with some sense of urgency given the widespread use among the general population. These studies generally involved the more commonly used products and assessed their influence on major metabolic enzyme systems. Perhaps not surprisingly, extensive lists of suspected botantical drug interactions have been generated based upon speculation based on specific phytoconstituents present in these products, the majority of which have yet to be documented in any convincing manner [8, 9]. However, a critical review of widely cited interactions purported to occur between botanical supplements and conventional medications reported that <15% were adequately documented, and thus, these lists are of questionable validity [10]. Whether the clear influence of SJW represents the tip of the iceberg, or perhaps the tip of the ice cube may be open to debate as few botanical supplements assessed to date have matched SJW in terms of its magnitude of effect on multiple substrates both *in vitro* and *in vivo*, and the replication of these effects. Coxeter and associates [11] recently raised the possibility of more "over reaction" than "interaction" regarding botanical-drug interactions. Although many normal volunteer botanical-drug interaction studies have been performed and published, *in vitro* investigations assessing both potential for interaction as well as mechanisms by which various natural products may interact with metabolic enzymes and transporters account for the majority of research performed and published to date.

There are many commonly used methods to assess drug-drug interactions *in vitro*. These methods have strengths as well as limitations, and their use in screening conventional drugs has been more extensively reviewed elsewhere [12, 13]. This mini-review is intended to focus more on the particular problems and challenges in evaluating botanical supplements using *in vitro* methods, and what can and cannot be learned from such investigations.

2 Mechanisms of drug interactions

Drug interactions are generally classified as pharmacodynamic or pharmacokinetic. This review focuses mainly on pharmacokinetic interactions which occur when absorption, distribution, metabolism or elimination are altered by another drug to produce the interaction. The primary route of drug elimination from the body is via biotransformation of the agent catalyzed by one or more enzymes [14]. Oxidative processes largely mediated by CYP450 enzymes are often broadly viewed as "functionalization" reactions said to represent phase I metabolism. In humans, approximately 60 active genes encode for various CYP450s of which CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5 are considered the major enzymes relevant to the majority of conventional medications. Other phase I processes include metabolic reduction, hydrolysis, and hydration. Conjugation reactions (phase II metabolism) typically follow phase I metabolism and involve the introduction of a hydrophilic endogenous species to the drug molecule, ultimately producing a more polar molecule which is more readily excreted in bile feces, or urine. Glucuronidation is one such major pathway catalyzed by one of many uridine diphosphoglucuronosyltransferases (UGTs). Other examples of phase II metabolism include sulfation, methylation, acetylation, and glutathione conjugation. There are a significant number of drug-metabolizing enzyme families and superfamilies which may exhibit overlapping substrate specificities [13].

Enzymes involved in phase I reactions are primarily located in the endoplasmic reticulum of hepatic cells. Enzymes involved in phase II reactions are mainly located in the cell cytosol, except UGTs, which are located in the endoplasmic reticulum [14]. Additionally, drug transporters such as P-glycoprotein (P-gp) are increasingly appreciated with regard to their important roles in the disposition of many drugs, drug interactions, and genetic variability [15]. Finally protein binding may also play a role in drug interactions although few clinically important interactions have been documented to occur by this mechanism [16].

The two major mechanisms of drug interactions involving enzymes and/or transporters are metabolic inhibition and induction. Metabolic inhibition can occur with almost all enzymes and transporters. Inhibitory effects on a specific drug metabolizing system can lead to toxicity as a result of increased plasma concentrations of the substrate compound and may occur as early as the first dosages administered depending on the agents involved. Toxicity may also occur if a previously minor metabolic pathway becomes favored, resulting in a toxic metabolite. Enzyme

inhibition can also result in reduced clinical efficacy if the substrate is a prodrug [12]. The current consensus holds that studying inhibition of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 enzymes is most important from a clinical perspective.

Conversely, CYP induction increases drug clearance which may lead to therapeutic failure [7, 17]. Metabolic induction does not occur with all enzymes and transporters, and has been most notably of concern for CYP3A, CYP2B6 and CYP1A2 as well as P-gp. With transporters, inhibition and induction can lead to both an increase or decrease in plasma/active site concentrations depending upon the direction of transport with respect to the active site of the drug and the type of transporter affected. Thus prediction of drug interactions through changes in transport interaction must take into consideration the site and direction of transport with respect to the individual drug and active site.

3 *In vitro* methods of predicting drug interactions: Strengths and weaknesses

The investigation into the drug-drug interaction potential of a new chemical entity which may be a candidate for further development has been a long-standing area of concern and investigation. Studies are typically carried out utilizing high-throughput *in vitro* study paradigms to assess compounds for drug-drug interaction potential. The US Food and Drug Administration has recently reported an increase in the inclusion of *in vitro* data in New Drug Applications and acknowledges the importance of this data in evaluating New Drug Applications and eventual product labeling [18]. Thus, the use of *in vitro* studies is clearly a valuable tool in drug development continuing to evolve in both methodology and interpretation. Although *in vitro* screening methods are both standard and accepted procedures in research and development efforts as well as post-marketing evaluation of conventional medications within the scientific community and pharmaceutical industry, a number of limitations are recognized in both the manner in which data are generated and the extrapolation of such data to the *in vivo* situation depending on the compounds studied, metabolic pathway assessed, and system utilized [12, 13].

Preclinical evaluation of drug metabolism and disposition as well as drug interaction assessments utilize a variety of *in vitro* enzyme and drug transporter sources and methodologies including microsomes, liver homogenates, cDNA expressed individual CYPs, or more purified enzyme preparations, primary hepatocytes, liver slices, and immortalized cell lines. Often cell lines that are deficient in or overexpress a specific transporter such as P-gp may be used [19]. Although these methods are clearly more rapid save and less expensive than clinical studies, inherent limitations exist in both the methods and the utilization of *in vitro* data. These include arbitrary assignment of concentration at the enzymatic site, and difficulty accounting for first-pass metabolism and active metabolites [12]. As stated in the introduction, these systems all have their relative strengths as well as limitations, and their use in screening conventional drugs has been more extensively reviewed elsewhere [12, 13].

4 Limitations of *in vitro* methods when assessing botanical products

Botanical products contain a wealth of naturally occurring phytochemicals including alkaloids, xanthines, coumarins, terpenes, steroids, lipids, carotenoids, flavonoids, isoflavones, isothiocyanates, phenolic, cinnamic and amino acids [20]. This list is by no means exhaustive and each class encompasses a wide variety of derivatives and isomers. It would be virtually impossible to perform clinical studies with individual constituents. Thus, screening with *in vitro* methodologies has been a feasible method of predicting which botanicals may be involved in interactions and should be examined clinically. However, the general limitations of *in vitro* studies which are briefly noted above require additional caveats unique to the nature of these multi-constituent, non-standardized products. An overview of limitations of the *in vitro* study of botanical supplements is presented in Table 1.

The selection of concentrations of botanical supplements to assess in *in vitro* study systems is substantially more problematic than that of assessing conventional compounds. The assignment of hepatic concentrations for compounds with thoroughly characterized pharmacokinetics is a well- known limitation of *in vitro* studies. With botanical products, there is a dearth of pharmacokinetic data on any but the most studied product and even these are product and dose specific, typically measuring only a few "marker" constituents characteristic of the product. These may or may not represent the purported active constituents. An accurate assessment of pharmacokinetic data is particularly problematic at present given the lack of comprehensive human disposition studies using well characterized botanical supplements. Of the small number of studies that are available for review, overall conclusions are limited for a number of reasons including use of different formulations, different or unknown dosages, and differences in what is measured analytically.

A problem frequently encountered in the existing *in vitro* literature is the use of inappropriately high concentrations of single isolated constituents obtained from commercial sources and utilization of these in experiments when only a small fraction of the compound may actually be bioavailable. Most natural products are generally subject to first-pass metabolism and to a much larger extent than conventional pharmaceutical agents which are in most cases specifically developed to be substantially bioavailable or other-

Table 1. General limitations and advantages of *in vitro* botanical-drug interaction studies

Limitations	– Limited or unknown absorption or bioavailability of the botanical constituents to their site of action – Uncertainty regarding clinically relevant concentrations of free compound *versus* conjugates or other metabolites formed *in vivo* – Metabolites of botanical extracts are poorly characterized for most extracts and may contribute to the net inhibitory or inductive effects observed – Single constituent often used in testing which are not reflective of typical multi-constuent extracts which may contribute of the net inhibitory or inductive effects observed – There is a known large product to product variability and known difficulties in characterization and standardization of products with complex phytochemical profiles. This may lead to difficulties in reproducibility of experiments
Advantages	– Relatively easy to perform in most laboratories investigating drug interactions – Inexpensive in comparison to clinical trials – Specific mechanisms can be evaluated under carefully controlled conditions

wise formulated as prodrugs. In addition, many are less bioavailable due to their hydrophilic nature or large molecular size. A number of recent studies assessing the biological activities of many phytochemical constituents of botanical products *in vitro* have been criticized due to the failure of investigators to acknowledge that these constituents do not exist when the native compounds are given *in vivo* but rather are present as conjugated metabolites that are formed primarily in the small intestine [21 – 24]. Since the unconjugated compounds do not ever reach the liver in any appreciable quantity, *in vitro* investigations to assess and predict the effects of these compounds on hepatic metabolism and transport are inaccurate[25].

The role of metabolites is almost entirely unknown for most supplements. The potential for unknown or univestigated constituents or metabolites to influence a given enzyme or transporter of interest cannot be excluded from consideration. Further, the limited commercial availability of many phytochemicals precludes their initial screening using *in vitro* systems. Even if single constituents or metabolites were available, examining them as individual entities *in vitro* would unlikely be representative of the *in vivo* situation following ingestion of these supplements. Natural products are generally a mixture of numerous agents which are often structurally related and have similar biological properties. When consumed in a mixture they may have additive or antagonistic activities which may not be apparent when an entity is tested in isolation.

Thus, there appear to be two large interrelated issues. Using high dose single constituents when in fact the botanical extract is a complex mixture results in the *in vitro* system being exposed to an exaggerated and unrealistic concentration. The second issue is that if hepatic enzymatic activity (or exposure anywhere requiring access to the systemic circulation) is of interest, the issue of bioavailability needs to be considered, independent of first pass potential. If a compound accesses the portal circulation and is thus exposed to the liver, hepatic enzymes are theoretically vulnerable to whatever activity the compound might have. Extensive metabolism might result in low amounts of the compound reaching the central compartment. If there are other issues such that the compound never gets through the intestinal wall, the only enzymes or transporters that might be affected would be intestinal (*e. g.* CYP3A, P-gp).

The highly variable nature of botanical supplements with regard to content of specific constituents, variability between what are ostensibly the same products with regard to quality and recommended dosage, dose frequency, and duration of regimens make extrapolations of *in vitro* findings to the *in vivo* situation particularly difficult [11].

The composition of botanical products is dependent upon agricultural practices, climactic conditions, post-harvest storage conditions as well as the particular extraction and processing conditions that create the final product [26]. For example a 13% ethanol extract of grapeseeds will produce a significantly different profile of constituents than a 100% acetone extract of the same grapeseeds. The extract composition will also differ if the seeds were waste products from winemaking which have already been extracted for many of the smaller molecular weight bioavailable constituents [27]. In the case of the dietary supplement industry in which little manufacturing oversight is required, this could prove to be yet another source of variability leading to difficulties in interpretation of data. Finally, when the extract is to be used in an *in vitro* study, researchers may arbitrarily dissolve the product in a solvent or buffer which may contain DMSO, ethanol or other organic solvent. The solubility of the constituents is likely to further contribute to experiment variability. The potential influence of some excipients (*e. g.* sorbitol) is increasingly appreciated with conventional pharmaceuticals because of their apparent ability to alter the bioavailability of certain medications [28].

All of the aforementioned limitations must be carefully considered for appropriate interpretation of *in vitro* studies in extrapolating to interactions in humans. Nonetheless, *in vitro* studies still provide much value in identifying specific botanical products or constituents that *may* potentially pose an interaction risk and thus serve as a "signal" that *in vivo* studies may be warranted to confirm clinical relevance.

5 *In vitro versus in vivo*: What have we learned?

Suspicions over the potential for specific botanical supplements and extracts to participate in clinically significant

drug interactions were validated in the year 2000 with two small, albeit high-profile reports appearing in the same issue of *The Lancet* that year. Both involved the putative botanical antidepressant SJW, *Hypericum perforatum*, which at the time enjoyed status as one of the top-selling supplements in the US. The first paper, a short research report by Piscitelli and coworkers (2000) described the results of a normal volunteer pharmacokinetic study (n = 16) in which blood concentrations of the HIV-1 protease inhibitor, indinavir were drastically reduced with SJW co-administration [7]. The second paper comprised two well documented case reports describing acute rejection reactions in transplant patients due to an interaction of SJW and cyclosporine resulting in precipitous falls in critical concentrations of the immunosuppressant [17]. Both cases suggested that one or more components in orally administered SJW supplements could cause clinically significant metabolic induction of CYP3A, and involved medications of major therapeutic importance. Numerous other credible reports soon followed. An exhaustive review of SJW interactions was published by Mannel in 2004 [29].

Until these reports had appeared, the primary drug interaction concerns with SJW were pharmacodynamic in nature and involved the theoretical potential for precipitating serotonin syndrome in individuals taking serotonergic medications (*e.g.* serotonin selective reuptake inhibitors) concomitantly with SJW due to the reported inhibitory effects of some SJW constituents on monoamine oxidase. Ensuing mechanistic studies identified the specific SJW constituent hyperforin as producing CYP3A induction via activation of a nuclear steroid/pregnane and xenobiotic receptor (SXR/PXR) in a concentration-dependent manner [30]. Further, there was substantial overlap between SJW-induced induction of CYP3A and induction of P-gp [31]. Thus, numerous credible case reports as well as systematic *in vitro* and *in vivo* studies soon followed suggesting significant periods of SJW exposure resulted in potent induction of both CYP3A and P-gp producing alterations in the disposition of numerous substrates [29].

The discrepancies between *in vitro* and *in vivo* drug interaction studies are exemplified by SJW which was first thought be an *inhibitor* of CYP3A4 due to its activity *in vitro* experiments [32], but is now known to be a potent *inducer* in clinical studies [7, 17, 33, 34]. Milk thistle (*Silybum marianum*) and its components silibinin and silymarin, were shown to alter the metabolism of CYP3A substrates *in vitro* [35, 36] but milk thistle was later shown to have no apparent activity in a clinical study [37]. Backman and colleagues [38] have also suggested a lack of correlation between *in vitro* and *in vivo* studies based upon their extensive work with the flavonoid, tangeretin. Other high-throughput approaches including the use of *in silico* or computational models have been increasingly utilized to evaluate the potential dispositional characteristics of even theoretical compounds. Although *in vitro* approaches are clearly more rapid and less expensive than clinical studies, and may generate an early signal of drug interaction potential or provide insight into the mechanistic aspects of drug-drug interactions, inherent limitations are recognized in all systems. These include perhaps most prominently the difficulties in the assignment of hepatic concentrations reflective of likely *in vivo* scenarios due to a variety of sources of variability, and difficulty accounting for first-pass metabolism, the potential production and contribution of metabolically active metabolites and others. An extensive review of this topic is beyond the scope of the present paper but several comprehensive reviews are available [12, 13, 39]. The *in vivo* concentration of a given inhibitor at an active or modulatory site is generally estimated and not known in *in vitro* experiments and typically based upon available pharmacokinetic values such as unbound or free concentration of the compound in plasma with the assumption that it is this concentration presented to hepatocytes and CYP or other enzymes or transporters [13]. Other investigators have suggested using portal vein concentrations although definitive these concentrations are not always available [40].

The purported hepatoprotective botanical supplement milk thistle (*Silybum marianum*) provides an excellent example of the disparate and conflicting results noted between *in vitro* investigations and *in vivo* findings in both animal models and controlled clinical studies. *In vitro*, silymarin extracts do not appear to inhibit CYP2E1 [41]. However, several other *in vitro* studies using human microsomes and hepatocytes have suggested potentially significant *inhibition* of CYP3A CYP2C9 [35, 42, 43] by components within the purported active extract of milk thistle seeds, silymarin, when studied at concentrations thought to be relevant to those possibly attained *in vivo*. Silymarin is composed of a mixture of flavonolignans including silybin (also called silibinin), isosilybin, and others. Additionally, there is *in vitro* evidence of inhibition of the phase II (conjugative) enzymes UGT 1A6/9/1 [42, 43] and P-gp [44]. Furthermore, following the oral feeding of silybin to mice, significant *induction* of the phase II enzymes glutathione *S*-transferase and quinone reductase were noted in multiple tissues [45].

When studied clinically in at least two published studies performed in normal volunteers, the results appeared to exonerate milk thistle extracts from participating in any significant metabolic interaction involving CYP3A or P-gp. Piscitelli and associates [46] detected no interaction between milk thistle supplements containing Silimarin and the protease inhibitor and CYP3A/P-gp substrate, indinavir. Similar non-significant findings with a milk thistle supplement that employed a different study design were recently reported by DiCenzo and co-workers [47]. Additionally, milk thistle supplementation in normal volunteers (110 mg silymarins, twice daily) only reduced midazolam (CYP3A probe substrate) clearance by 13% [20] and was not found

to significantly alter CYP3A or P-gp activity in two subsequent volunteer studies [48, 49]. Thus, the potential for interactions mediated by CYP3A and/or P-gp is suggested by *in vitro* assessments, but has not been confirmed in numerous clinical studies utilizing known substrates of CYP3A and P-gp. To our knowledge there are no clinical studies that corroborate the *in vitro* findings that milk thistly alters the activity of Phase II enzymes *in vivo* as suggested by previous *in vitro* studies [45] although there is a paucity of clinical data that focuses specifically on Phase II enzyme activity.

The focus of this review was to highlight the challenges that face scientists when trying to predict drug interactions from *in vitro* experiments and not to imply that *in vivo* studies with botanical products have always contradicted the previous *in vitro* findings.

There are of course some examples where clinical studies confirmed the *in vitro* inhibitory effects human CYP3A4 and CYP2D6. One example is with the dietary supplement, goldenseal (*Hydrastis canadensis*) [50, 51]. In addition, the usefulness of using *in vitro* studies to subsequently determine the mechanism of action for a known drug interaction that was discovered clinically should also be noted. The mechanism of action for grapefruit/drug interactions has been clarified through a combination of elegant clinical and *in vitro* studies [1, 2, 52].

Ultimately, *in vivo* clinical studies are the most reliable means to determine the clinical importance of botanical-drug interactions. However, these studies too can be quickly confounded by the documented variability found in specific constituents between individual botanical products as well as the choice of probe substrates administered.

6 Conclusion

In vitro methods have been widely used in attempts to predict potential drug interactions with botanical products, but to date, few significant drug interactions have actually been discovered or accurately predicted with botanical products. Instead, much of the research has led to clinical studies with negative outcomes. While the exoneration of a given botanical product is an important finding and may produce some measure of comfort to clinicians, the expense and effort to perform these studies is not trivial.

In vitro studies utilized to assess drug interaction potential with natural products have all the limitations of *in vitro* studies with conventional drugs including assignment of hepatic concentrations, accounting for first-pass metabolism and active metabolites, and additionally, the lack of commercial standards, and the inability to accurately screen the entities as mixtures. These complications have contributed to the discrepancies between outcomes predicted by *in vitro* methods and observed *in vivo* effects. Accordingly, it may be time to re-evaluate the value of *in vitro* screening of individual phytoconstituents and extract mixtures and critically assess the clinical implications from *in vitro* studies that are conducted, with consideration to the many limitations.

The authors have declared no conflict of interest.

7 References

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