Clinical Pharmacokinetic Interactions between Herbal Supplements and Anticancer Drugs

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Clinical Pharmacokinetic Interactions between Herbal Supplements and Anticancer Drugs

Klinische farmacokinetische interacties tussen kruidensupplementen
en anti-kanker geneesmiddelen
(met een samenvatting in het Nederlands)

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"Life is like riding a bicycle.
To keep your balance, you must keep moving."
- Albert Einstein

Aan mijn ouders

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CHAPTER 1

General introduction

Due to wide interindividual variations in response to chemotherapy, many cancer patients may not be optimally treated. An anticancer drug that is effective in one patient can be subtherapeutic or more toxic in another patient with the same type of cancer. The use of complementary alternative medicines (CAM) by cancer patients may contribute to this serious health problem. Over the past decades the use of CAM by cancer patients has increased rapidly and about 10 to 95% of the cancer patients have reported to use CAM [1]. In general, the use of CAM is considered safe and non-toxic for patients. Given this belief and the fact that physicians are often unaware of CAM use by their patients, undertreatment or increased toxicities by chemotherapy is generally not related to the use of CAM.

Currently, little research has been executed to elucidate the clinical effects of CAM on the pharmacological activity of anticancer drugs. Consequently, for many CAM it is unknown whether concomitant use with anticancer drugs is safe. CAM, in particular herbal supplements, may compromise the therapeutic efficacy or safety profile of chemotherapy by interacting with conventional anticancer drugs. A recent systematic review article has shown that the majority of herb-drug interactions were attributable to pharmacokinetic-related mechanisms [2]. Therefore, this thesis focused on pharmacokinetic interactions between herbal supplements and anticancer drugs in cancer patients. Results of this thesis will aid in establishing guidelines regarding the use of herbal supplements by cancer patients.

Pharmacokinetic interactions between herbal supplements and anticancer drugs is the main subject of this thesis, since herbal supplements have been shown to affect cytochrome P450 (CYP) enzymes and drug transporters, such as P-glycoprotein (P-gp; ABCB1) and Breast Cancer Resistance Protein (BCRP; ABCG2). As CYP enzymes and drug transporters are involved in the metabolism and elimination of many anticancer drugs, inhibition or induction by CAM could alter the plasma concentrations of these agents. Considering the narrow therapeutic window of anticancer drugs, changes in plasma concentrations could easily have serious clinical consequences such as an increased risk of toxicities or a reduced therapeutic efficacy. For example, the herbal antidepressant St. John's wort (SJW) significantly decreased the area under the plasma concentration-time curve of irinotecan's active metabolite SN-38 [3], which was accompanied by less severe irinotecan-induced neutropenia and leukopenia after SJW supplementation.

According to the National Center for Complementary and Alternative Medicine the definition of CAM is relatively broad and comprises natural products (e.g. herbal medicines, dietary supplements), mind and body medicine (e.g. meditation, yoga, acupuncture) and manipulative and body-based practices (e.g. spinal manipulation, massage therapy). In

this thesis, however, the focus is on popular herbal supplements used in Western society [4]: *Echinacea purpurea*, St. John's wort, milk thistle and grape seed extract. Before conducting clinical interaction studies, the potential of herbal supplements to inhibit or induce drug metabolizing enzymes is often investigated *in vitro*. **Chapter 2.1** discusses the relevance of *in vitro* and clinical results for the prediction of pharmacokinetic herb-drug interactions in cancer patients. In **Chapter 2.2** the effect of the botanical compound mixture LCS101 on chemotherapy is commented.

Each subsequent chapter of this thesis describes the clinical effect of a single herbal supplement on the pharmacokinetics of an anticancer drug and/or a CYP probe substrate. **Chapter 3** focuses on the effects of the herbal immunostimulant *Echinacea purpurea* on the pharmacokinetics of the anticancer agent docetaxel in patients with advanced cancer. Docetaxel was chosen not only because it is an important and often applied anticancer agent, but also because it has a narrow therapeutic window and it is a good substrate drug for CYP3A as it is exclusively metabolized by this CYP enzyme. In support of this clinical interaction study (**Chapter 3.3**), two bioanalytical assays based on liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) have been developed and validated for the quantification of alkylamides in human plasma (**Chapter 3.1** and **3.2**), which are the major bioavailable constituents of *Echinacea purpurea*. Herbal supplements are often characterized by a large variation in phytochemical content. To address this issue, a quality control analysis has been performed for alkylamide content in several batches of a commercially available *Echinacea purpurea* liquid extract (**Chapter 3.4**).

SJW is known for its CYP3A4 inducing effects on the pharmacokinetics of several CYP3A4 substrates including the anticancer drugs imatinib and irinotecan. **Chapter 4.1** describes the pharmacokinetic interaction study between SJW and docetaxel, which is a more sensitive substrate for CYP3A4 than imatinib and irinotecan.

In **Chapter 5** the inhibiting effects of milk thistle on the metabolism of docetaxel (CYP3A4) and the sensitive CYP2C9 probe tolbutamide have been determined in cancer patients. Milk thistle, which is a popular herbal supplement among cancer patients, has been selected for this clinical trial to investigate the clinical significance of inhibition of CYP2C9 and CYP3A4 by milk thistle in *in vitro* studies. The interim analysis of this trial, which is still ongoing, is presented in **Chapter 5.1**.

In vitro data indicated that grape seed extract inhibits CYP2D6. Inhibition of CYP2D6 could be relevant for cancer patients using the antiestrogenic agent tamoxifen, which is extensively metabolized by CYP2D6. The clinical significance of CYP2D6 inhibition by grape seed is addressed in **Chapter 6.1**, which discusses the pharmacokinetic interaction

study between grape seed extract and the sensitive CYP2D6 substrate dextromethorphan in healthy volunteers. In this study dextromethorphan served as a model drug for tamoxifen.

In summary, this thesis describes the effects of the widely-used herbal supplements *Echinacea purpurea*, SJW, milk thistle and grape seed extract on the pharmacokinetics of substrates of CYP3A4 (docetaxel), CYP2C9 (tolbutamide) and CYP2D6 (dextromethorphan). Considering the paucity of clinical herb-drug interaction studies, the presented results contribute to the knowledge of (un)safe herb-drug combinations in cancer patients. Ultimately, this work will help physicians in advising their patients about concomitant use of herbal supplements and anticancer drugs.

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CHAPTER 2

Review clinical herb-drug interactions

CHAPTER 2.1

Relevance of *in vitro* and clinical data for predicting CYP3A4-mediated herb-drug interactions in cancer patients

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ABSTRACT

The use of complementary and alternative medicines (CAM) by cancer patients is increasing. Concomitant use of CAM and anticancer drugs could lead to serious safety issues in patients. CAM have the potential to cause pharmacokinetic interactions with anticancer drugs, leading to either increased or decreased plasma levels of anticancer drugs. This could result in unexpected toxicities or a reduced efficacy. Significant pharmacokinetic interactions have already been shown between St. John's wort (SJW) and the anticancer drugs imatinib and irinotecan.

Most pharmacokinetic CAM-drug interactions, involve drug metabolizing cytochrome P450 (CYP) enzymes, in particular CYP3A4. The effect of CAM on CYP3A4 activity and expression can be assessed *in vitro*. However, no data have been reported yet regarding the relevance of these *in vitro* data for the prediction of CAM-anticancer drug interactions in clinical practice. To address this issue, a literature research was performed to evaluate the relevance of *in vitro* data to predict clinical effects of CAM frequently used by cancer patients: SJW, milk thistle, garlic and *Panax ginseng* (*P. ginseng)*. Furthermore, in clinical studies the sensitive CYP3A4 substrate probe midazolam is often used to determine pharmacokinetic interactions. Results of these clinical studies with midazolam are used to predict pharmacokinetic interactions with other drugs metabolized by CYP3A4. Therefore, this review also explored whether clinical trials with midazolam are useful to predict clinical pharmacokinetic CAM-anticancer drug interactions.

In vitro data of SJW have shown CYP3A4 inhibition after short-term exposure and induction after long-term exposure. In clinical studies using midazolam or anticancer drugs (irinotecan and imatinib) as known CYP3A4 substrates in combination with SJW, decreased plasma levels of these drugs were observed, which was expected as a consequence of CYP3A4 induction. For garlic, no effect on CYP3A4 has been shown *in vitro* and also in clinical studies garlic did not affect the pharmacokinetics of both midazolam and docetaxel. Milk thistle and *P. ginseng* predominantly showed CYP3A4 inhibition *in vitro*. However, in clinical studies these CAM did not cause significant pharmacokinetic interactions with midazolam, irinotecan, docetaxel and imatinib. Most likely, factors as poor pharmaceutical availability, solubility and bioavailability contributed to the lack of significant clinical interactions. In conclusion, *in vitro* data are useful as a first indication for potential pharmacokinetic drug interactions with CAM. However, the discrepancies between *in vitro* and clinical results for milk thistle and *P. ginseng* show that clinical studies are required for confirmation of potential interactions. At last, midazolam as a model substrate for CYP3A4, has convincingly shown to correctly predict clinical interactions between CAM and anticancer drugs.

INTRODUCTION

The use of complementary and alternative medicines (CAM) is popular among cancer patients. For example, 75% of colorectal cancer patients [1], 90% of prostate cancer patients [2] and 91% of pediatric cancer patients [3] have been reported to use CAM. The most popular CAM among cancer patients are herbal supplements and vitamins [4]. Cancer patients use these CAM in combination with their conventional therapies for different reasons, such as reducing side effects of chemotherapy, slowing progression of cancer, and improving quality of life [5,6]. The concomitant use of anticancer drugs and CAM, however, could lead to serious safety issues in cancer patients, as CAM have the potential to cause pharmacokinetic and -dynamic interactions with anticancer drugs [4, 7-9]. Especially for anticancer drugs, which have narrow therapeutic windows, pharmacokinetic interactions could easily lead to clinically relevant effects. Decreased or increased plasma levels of these drugs could result in a lower therapeutic efficacy or a higher risk of toxicity [4, 5]. Clinically relevant pharmacokinetic interactions between anticancer drugs and CAM have already been reported between the frequently used CAM St. John's wort (SJW) and the anticancer drugs irinotecan [10] and imatinib [11, 12].

Almost all pharmacokinetic CAM-anticancer drug interactions involve cytochrome P450 (CYP) metabolizing enzymes, drug transporters (e.g. P-glycoprotein), and other metabolic pathways [6]. The majority of currently prescribed anticancer drugs are metabolized by CYP3A4 [13]. For anticancer drugs such as docetaxel, erlotinib, imatinib, irinotecan, paclitaxel and vincristine, CYP3A4 is the main CYP enzyme involved in their metabolism. Several *in vitro* and clinical studies have been performed to determine the effects of CAM on CYP3A4. However, no data have been reported regarding the predictive value of these *in vitro* data for clinical CAM-anticancer drug interactions. Therefore, the main focus of this review is to determine the relevance of *in vitro* data to predict clinical pharmacokinetic CAM-anticancer drug interactions. To address this issue, a literature research was performed to evaluate *in vitro* and clinical effects on CYP3A4 by frequently used CAM: SJW, milk thistle, garlic and *Panax ginseng* (*P. ginseng*). This selection is based on the high frequency of use by cancer patients and the availability of both *in vitro* and clinical data regarding pharmacokinetic CAM-drug interactions. As there are large interspecies differences in the regulation of CYP3A expression and activity [14], animal data are not included in this review. In clinical studies, the sensitive CYP3A4 substrate probe midazolam is often used to determine pharmacokinetic interactions [15]. The second focus of this review was therefore to assess the relevance of clinical trials with midazolam to predict clinical pharmacokinetic interactions between CAM and anticancer drugs.

2 METHODS TO INVESTIGATE PHARMACOKINETIC CAM-DRUG INTERACTIONS

The following chapters will provide an overview of *in vitro* and clinical effects of CAM on CYP3A4 activity and expression. To facilitate the interpretation of these *in vitro* and clinical results, the methods to determine *in vitro* and clinical pharmacokinetic interactions will be described briefly.

In vitro inhibition assays of CYP3A4

To determine the inhibiting effect of CAM on CYP3A4 activity, different *in vitro* test systems can be used: cultured cell lines, human liver microsomes (subcellular fractions of a human liver consisting of smooth endoplasmic reticulum vesicles containing the main CYP enzymes) [16,17], Supersomes™ (human lymphoblast cells containing an overexpression of CYP enzymes) [18] and primary human hepatocytes [19]. The use of microsomes and Supersomes™ is preferred and recommended by the Food and Drug Administration (FDA) [20-22]. Compared to cell lines and hepatocytes, microsomes and Supersomes™ are commercially available and the results obtained by these test systems are not confounded by cellular uptake or other intracellular metabolic processes [20]. A drawback of microsomes and Supersomes™ is that they do not completely mimic the physiological hepatic environment [20]. The ability of CAM to inhibit CYP3A4 activity is determined by treating cells, microsomes, Supersomes™ or hepatocytes with a CYP3A4 substrate in the absence or presence of CAM or potent CYP3A4 model inhibitors (preferably ketoconazole [20]). The amount of CYP3A4 inhibition is established by determining changes in the metabolism of model substrates of CYP3A4 by monitoring the decrease of metabolite formation. For quantification of the metabolites, two standard assays are frequently used: liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) or a fluorescence assay. Although a fluorescence assay is fast, cost-effective and sensitive, LC-MS/MS is preferred [23], because of the higher sensitivity and specificity [24]. Other disadvantages of the fluorescence assay are the possible interference of fluorescence (quenching) by CAM and inadequate selectivity of many CYP substrate probes. These substrate probes are often also metabolized by other CYP enzymes, besides CYP3A4. Recombinantly expressed single CYP enzymes (Supersomes™) can be used to overcome this selectivity problem [23]. For LC-MS/MS, preferred CYP3A4 substrates are midazolam and testosterone [20]. For the fluorescence assay, four fluorescence substrates have been reported useful in screening CYP3A4 inhibition: 7-benzyloxyquinoline (BQ), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), dibenzylfluorescein (DBF) and 7-benzyloxyresorufin (BzRes). BFC and DBF are preferred substrates, as the incubation time and total amount of CYP3A4 required are lower compared to BQ and BzRes [25]. The amount of CYP3A4 inhibition is usually expressed as IC_{50}, the concentration required to achieve 50% of the maximum inhibition.

It is important to realize that CYP3A4 inhibition is system-dependent [21]. Different test systems deviate in their interaction with CAM, which can result in under- or overprediction of CYP3A4 inhibition. Therefore, it is recommended to combine multiple test systems and methods of quantification to confirm CYP3A4 inhibition by CAM. However, in the majority of the reported *in vitro* studies in literature only one test system was used.

In vitro induction assays of CYP3A4

Besides inhibition of CYP3A4 by CAM, CAM can also induce this CYP enzyme by affecting the gene expression of CYP3A4. CYP3A4 gene expression is mainly regulated by the nuclear pregnane X receptor (PXR) [26]. Activation of PXR results in transcription of the CYP3A4 gene and CYP3A4 induction. Primary human hepatocytes are the most accepted and reliable *in vitro* test system to evaluate CYP3A4 induction by CAM. However, major drawbacks are high costs, poor availability and rapid decrease in CYP expression [22, 27]. Alternatively, cultured cell lines are commonly used, such as the human hepatocarcinoma derived cell line HepG2, the human colon carcinoma derived cell line LS180, [27] and a novel primary human hepatocyte clone, the Fa2N-4 cell line. Disadvantages of the use of cell lines compared to primary hepatocytes are: lower expression of CYP enzymes, lower basal enzyme activities and absence of all phenotypic characteristics of human hepatocytes, such as receptor function and expression [23].

To determine CYP3A4 induction by CAM, hepatocytes or cell lines are treated with CAM or a model inducer (preferably rifampicin [20]). The amount of CYP3A4 induction can be quantified by two frequently used standard assays: detecting mRNA levels using real-time polymerase chain reaction (RT-PCR) and the protein expression assay using Western blotting. However, it is complicated to demonstrate CYP3A4 induction at the level of gene and protein expression, due to low levels of CYP3A4 in cell lines and the rapid decrease in CYP3A4 levels in hepatocytes [27]. An assay without these limitations is the reporter gene assay. In this assay, transfected cell lines are used in which a luminescent signal can be measured proportional to PXR-mediated CYP3A4 gene induction [20, 22, 27]. Despite the fact that PXR is artificially increased in this assay, CYP3A4 induction measured by the reporter gene assay has been shown to correlate with CYP3A4 gene and protein expression in human hepatocytes [27].

Changes in CYP3A4 gene or protein expression do not necessarily lead to changes in the activity of this enzyme. Therefore, it is also important to determine the effect of CAM on CYP3A4 activity which is mostly done with LC-MS/MS or a fluorescence assay as described above [20].

Overall, similar to the determination of CYP3A4 inhibition it is also important to confirm CYP3A4 induction by CAM with multiple test systems and methods of quantification.

2

Clinical pharmacokinetic interaction studies

If *in vitro* data reveal significant inhibition or induction of CYP enzymes by CAM, clinical studies are often conducted to determine the presence of pharmacokinetic interactions in humans. Several factors are involved in the execution of clinical interaction studies, such as study design, chosen probe drugs, and pharmacokinetic endpoints.

To compare plasma concentrations of CYP3A4 substrate (S, midazolam) with and without interacting drug (I, CAM) the following study designs can be applied: randomized crossover (S followed by S + I, S + I followed by S), one-sequence crossover (S followed by S + I (or reversed)) and a parallel design (S in group 1, S + I in group 2) [22]. In each of these study designs midazolam plasma concentrations with and without CAM are compared [22]. Of these study designs, a randomized crossover design should be preferred over a one-sequence design to correct for sequence, period and carry-over effects [28]. The majority of the clinical studies described in this review have applied a one-sequence crossover design.

The most sensitive CYP substrates should be selected for clinical interaction studies. A CYP substrate is considered "sensitive" when its area under the plasma concentration-time curve (AUC) increases ≥ 5-fold when co-administered with a potent CYP inhibitor [22]. For CYP3A4, oral midazolam is a recommended sensitive substrate [22], as CYP3A4 plays a major role in the biotransformation of midazolam to its metabolites 1-hydroxy- and 4-hydroxymidazolam [29].

After selection of the study design and a sensitive CYP3A4 substrate, an appropriate pharmacokinetic endpoint should be chosen. Frequently, pharmacokinetic exposure parameters such as the AUC and the maximum plasma concentration (C_{max}) are main endpoints. Occasionally, the pre- and postsupplementation phenotypic ratio of 1-hydroxymidazolam to midazolam is determined [30, 31], which provides a useful estimate of midazolam clearance [32]. The AUC however, more accurately reflects systemic drug exposure and is therefore a recommended pharmacokinetic parameter for drug interaction studies [17, 22]. Strong inhibitors of CYP3A4 will cause ≥ 5-fold increase of midazolam AUC, while moderate (≥ 2- and < 5-fold increase) or weak (1.25- and < 2-fold increase) CYP3A4 inhibitors will cause less significant elevations of the AUC of midazolam.

As anticancer drugs and midazolam may differ in their affinity for CYP3A4, interaction studies with CAM and anticancer drugs have to confirm the results obtained by midazolam. By comparing the results of CAM with midazolam and anticancer drugs, it is possible to determine the predictive value of midazolam as model substrate for pharmacokinetic interactions.

COMPARISON BETWEEN *IN VITRO* AND CLINICAL DATA ABOUT CAM-DRUG INTERACTIONS

The following paragraphs provide an overview of the *in vitro* and clinical effects of popular CAM (St. John's wort, milk thistle, garlic and *P. ginseng*) on CYP3A4. For each CAM the predictive value of *in vitro* data for the clinic will be discussed. Additionally, the clinical use of the CYP3A4 probe midazolam to predict CYP3A4-mediated interactions between CAM and anticancer drugs in humans will be assessed.

St. John's wort

St. John's wort (*Hypericum perforatum*, SJW), used for treatment of mild to moderate depression [33], is a popular herbal medicine among cancer patients [10]. According to a survey among cancer patients, SJW belongs to the top 10 of used CAM [6].

In vitro effects of SJW on CYP3A4 and drug metabolism

Several *in vitro* studies have been performed to determine the effects of SJW and its main components hyperforin, hypericin and quercitrin on CYP3A4 (Table 1A) [33]. CYP3A4 mediated metabolism of midazolam and testosterone was inhibited by hyperforin, hypericin and I3-II8-biapigenin in microsomes [34, 35]. SJW extract inhibited CYP3A4 activity in Supersomes™ [36]. Hypericin also inhibited CYP3A4 activity in baculosomes (insect cells containing an overexpression of CYP enzymes [18]) and CYP3A4 metabolism of cortisol in microsomes [37]. Reviewing these *in vitro* studies, CYP3A4 inhibition by SJW has been shown in the preferred test systems (microsomes or Supersomes™) and should be considered as relevant. The IC_{50} values (0.038 – 17.2 µM) of SJW main components are comparable to an average IC_{50} value (0.03 µM) of the potent CYP3A4 inhibitor ketoconazole [38]. Besides CYP3A4 inhibition by SJW also CYP3A4 induction was shown in multiple *in vitro* studies. In LS180 cells hypericin, hyperforin and quercitrin induced CYP3A4 mRNA expression [33]. Accordingly, the inducing effect of SJW extract and hyperforin on CYP3A4 mRNA expression has been demonstrated in hepatocytes [26, 39]. Hyperforin also induced CYP3A4 protein expression and activity and increased the metabolism of docetaxel in hepatocytes [39, 40]. Induction of PXR-mediated CYP3A4 expression by the standardized SJW extract has been demonstrated in LS180 cells using a CYP3A4 reporter gene assay by our research group [26]. The extent of CYP3A4 induction by SJW and its main component hyperforin is comparable to the induction by the potent inducer rifampicin [26, 33, 39] and should therefore be considered as relevant.

Overall, both CYP3A4 inhibition and induction by SJW and its main components has been demonstrated. Whether inhibition or induction occurs, may depend on the duration of incubation with SJW. Inhibition of CYP3A4 by SJW was observed with incubation times of 10-60 min [26, 33, 37, 39, 40], whereas induction was found after incubation with SJW for at least

Table 1A. Effect of SJW on the activity of CYP3A4 *in vitro*

Models	CAM/component	SJW product	Assay	Results	Ref
LS180 cells	10 µM hyperforin 10 µM hypericin 10 µM quercitrin	Phytoplan, Heidelberg, Germany Sigma, Buchs Switzerland Extrasynthèse, Genay, France	RT-PCR	7 times ↑ mRNA 11 times ↑ mRNA 7 times ↑ mRNA	33
Human hepatocytes	1 µM hyperforin and 7-75 µg/mL SJW extract	Apin Chemicals Limited, Abingdon, UK 1. Nature's Way Products, Springville. USA 2. Nature's Plus, Melville, USA 3. Nutraceutical, Park City, USA	Northern blotting	6-7 times ↑ mRNA	26
Human hepatocytes	1-2.5 µM hyperforin	Purified from SJW leaf/flower mixtures and identified	RT-PCR, Western blotting and HPLC-UV (testosterone)	↑ mRNA, protein, CYP3A4 activity (by hyperforin)	39
Human hepatocytes	0.2-1 µM hypericin	Sigma-Aldrich, St. Louis, USA	RT-PCR, Western blotting and HPLC-UV (testosterone)	↔ mRNA, protein, CYP3A4 activity (by hypericin)	39
Human hepatocytes	0.1-1.5 µM hyperforin	Purified from SJW leaf/flower mixtures and identified	LC-MS/MS (docetaxel)	2.6-7 times ↑ CYP3A4 activity	40
Human liver microsomes	0.1-100 µM hyperforin	Phytolab GmbH, Vestenbergsgreuth, Germany	LC-MS (midazolam and testosterone)	↓ CYP3A4 activity IC_{50} = resp. 9.6 µM and 8.4 µM	34
Human liver microsomes	hypericin hyperforin, I3-II8-biapigenin	BIOMOL, Plymouth Meeting, USA Isolated and purified from commercial SJW products: Centrum Herbals, Madison or Quanterra, Morris Plains, USA	HPLC-MS (testosterone)	↓ CYP3A4 activity K_i = resp. 4.2; 0.49 and 0.038 µM	35
Rat liver microsomes	50 µM hypericin	Sigma Chemical Co., St. Louis, USA	HPLC-UV (cortisol)	35% ↓ CYP3A4 activity	37
Baculosomes	50 µM hypericin	Sigma Chemical Co., St. Louis, USA	Fluorescence assay (Vivid red)	↓ CYP3A4 activity IC_{50} = 868 nM	37
Supersomes™	0.39-850 µg/mL SJW extract	Hypericum Stada®, Bad Vilbel, Germany	Fluorescence assay (BQ and BFC)	↓ CYP3A4 activity IC_{50} = resp. 10.3 and 11.8 µM	36
Supersomes™	8-400 µg/mL SJW extract	Hypericum Stada®, Bad Vilbel, Germany	HPLC-UV (testosterone)	CYP3A4 activity IC_{50} = 17.2 µM	36

Abbreviations: RT-PCR = real-time polymerase chain reaction; HPLC-UV = high-performance liquid chromatography with ultraviolet detection; LC-MS = liquid chromatography mass spectrometry; BQ = 7-benzyloxyquinoline; BFC = 7-benzyloxy-4-trifluoromethylcoumarin; IC_{50} = half maximal inhibitory concentration; K_i = *in vitro* inhibition constant. ↔ = unchanged, ↓ = decrease and ↑ = increase.

30-96 h [34-36]. Inhibition after short-term incubation could be explained by competitive inhibition [7], as hyperforin has been reported as a competitive inhibitor of CYP3A4 [35]. Induction is expected after prolonged exposure, as the SJW constituent hyperforin activates PXR-mediated CYP3A4 gene transcription and causes CYP3A4 induction [26]. In clinical practice, SJW is generally administered for a prolonged period of time and therefore CYP3A4 induction is more likely to occur than inhibition.

Clinical pharmacokinetic interactions between SJW and midazolam

Several studies have been reported about clinical pharmacokinetic interactions between SJW and midazolam in healthy volunteers (Table 1B). In these studies a one-sequence crossover design was applied: midazolam administration was followed by SJW supplementation after which subjects received the second administration of midazolam. In accordance with the *in vitro* data, induction of CYP3A4 after supplementation with SJW (300 - 500 mg extract two or three times daily) has been observed in all of these studies, regardless of the pharmacokinetic endpoint (AUC, clearance, phenotypic ratio), brand of SJW extract, duration of SJW supplementation (10 days - 8 weeks), subjects' age or ethnicity.

Interestingly, SJW has a more potent inductive effect on intestinal CYP3A4 than on CYP3A4 located in the liver. This has been shown by studies administering both oral and intravenous (IV) midazolam: the increase of clearance of midazolam was greater after oral administration [41-44]. This finding suggests that oral CYP3A4 substrates (both intestinal and hepatic CYP3A4 involved) are more susceptible to CYP3A4 induction by SJW than IV substrates (predominantly hepatic CYP3A4 involved). Furthermore, the magnitude of CYP3A4 induction by a SJW product depends on its hyperforin content [45], which has shown to be the main SJW constituent responsible for induction in *in vitro* studies [26, 33, 39]. This has been demonstrated by a study in which a SJW product with low hyperforin content did not significantly decrease the AUC of midazolam [46].

Overall, clinical interaction studies with SJW and midazolam showed that SJW significantly induced CYP3A4 (primarily intestinal CYP3A4), resulting in a decreased bioavailability of the orally taken model substrate midazolam. Thus, *in vitro* results demonstrating CYP3A4 induction by SJW [26, 33, 39] were extrapolatable to the clinical setting with midazolam.

Clinical pharmacokinetic interactions between SJW and anticancer drugs

For cancer patients it is important to be aware of potential pharmacokinetic interactions between SJW and anticancer drugs. Based on the interaction between SJW and midazolam, pharmacokinetic interactions with anticancer drugs metabolized by CYP3A4 could be expected. Thus far, the effect of SJW on the pharmacokinetics of anticancer drugs has been investigated for irinotecan [10] and imatinib [11, 12] (Table 1B). In a randomized

Table 1B. Effect of SJW on the pharmacokinetics of selected substrate drugs in healthy volunteers and patients

Subjects	Dose SJW	SJW product	Substrate	Results	Ref
Healthy volunteers (*n* = 12)	300 mg (0.3% hypericin), thrice daily, 14 days	Sundown Herbals, Boca Raton, USA	MDZ (P.O., IV)	52% ↓ $AUC_{0-\infty}$ oral MDZ, 21% ↓ $AUC_{0-\infty}$ IV MDZ	41
Healthy volunteers (*n* = 12)	300 mg (0.3% hypericin), thrice daily, 8 weeks	Rexall-Sundown Pharmaceuticals, Boca Raton, USA	MDZ (P.O., IV)	53% ↑ CL oral MDZ, 3% ↑ CL IV	42
Healthy volunteers (*n* = 12)	300 mg, thrice daily, 14 days	TruNature, Leiner Health Products, Carson, USA	MDZ (P.O.)	33% ↑ CL oral MDZ, baseline CL MDZ restored 7 days after completion	85
Healthy volunteers (*n* = 12)	300 mg (0.3% hypericin), thrice daily, 28 days	Vitamer, Lake Forest, USA	MDZ (P.O.)	141% ↑ 1-OH MDZ / MDZ serum ratio	30
Healthy volunteers (*n* = 12)	300 mg (0.3% hypericin), thrice daily, 28 days	Wild Oats Markets, Inc, Boulder, USA	MDZ (P.O.)	98% ↑ 1-OH MDZ / MDZ serum ratio	31
Healthy volunteers (*n* = 20)	500 mg, twice daily, 14 days	Kneipp Werke, Würzburg, Germany	MDZ (P.O.)	15% ↓ $AUC_{0-\infty}$ oral MDZ	46
Healthy volunteers (*n* = 21)	300 mg (0.3% hypericin), thrice daily, 12 days	Jarsin 300 dragée, LI 160, Lichtwer Pharma AG, Berlin, Germany	MDZ (P.O., IV)	168% ↑ CL oral MDZ 44% ↑ CL IV MDZ	43
Healthy volunteers (*n* = 30)	300 mg, thrice daily, 10 days	Unknown	MDZ (P.O., IV)	190% ↑ CL oral MDZ 56% ↑ CL IV MDZ	44
Healthy volunteers (*n* = 42)	300-900 mg, twice/thrice daily, 14 days	1. Jarsin 300 dragée, LI 160, Lichtwer Pharma AG, Berlin, Germany 2A. Type A, Kneipp Werke, Würzburg, Germany 2B. Kneipp Johanniskraut Dragees H, Kneipp Werke, Würzburg, Germany	MDZ (P.O.)	↓ AUC oral MDZ (sign. correlation with hyperforin content)	45
Cancer patients (*n* = 5)	300 mg, thrice daily, 18 days	Bio Nutrition Health Products, Den Bosch, The Netherlands	Irinotecan (IV)	42% ↓ AUC SN-38, ↓ myelosuppression	10
Healthy volunteers (*n* = 12)	300 mg, thrice daily, 14 days	Kira, LI 160, Lichtwer Pharma AG, Berlin, Germany	Imatinib (P.O.)	30% ↓ $AUC_{0-\infty}$	12
Healthy volunteers (*n* = 10)	300 mg, thrice daily, 14 days	HBC Inc., Los Angeles, USA	Imatinib (P.O.)	32% ↓ $AUC_{0-\infty}$	11

Abbreviations: P.O. = oral administration; IV = intravenous administration; 1-OH MDZ = 1-hydroxymidazolam; MDZ = midazolam; CL = total plasma clearance; $AUC_{0-\infty}$ = area under the plasma concentration-time curve from time zero to infinity. ↓ = decrease and ↑ = increase.

crossover study five cancer patients were treated with irinotecan (350 mg/m^2, IV) and SJW [10]. In irinotecan's metabolism CYP3A4 mediates the formation of the parent drug to the metabolites APC and NPC. NPC is then converted by human carboxylesterase 2 to the active metabolite SN-38, which is subsequently glucuronidized to SN-38G by UDP glucuronosyl transferases. Drug transporters such as P-gp and Breast Cancer Resistance Protein are involved in the elimination of irinotecan [47]. In accordance to the CYP3A4 induction in midazolam studies, SJW significantly decreased the AUC of irinotecan's active metabolite SN-38 by 42%. Consequently, there was also a substantial difference in the degree of myelosuppression: the decrease of leukocyte and neutrophil counts was greater after irinotecan alone compared to the course with irinotecan and SJW combined. In a one-sequence crossover study including twelve healthy subjects, fourteen days of SJW intake also decreased systemic exposure to imatinib [12], which is mainly metabolized by CYP3A4 to the active *N*-desmethylated piperazine derivative (CGP74588). In the presence of SJW the $AUC_{0-\infty}$ of imatinib (400 mg/day) was significantly decreased by 30%. Similar results were observed in another one-sequence crossover trial with SJW and imatinib [11]. In this trial two weeks of SJW supplementation resulted in a significant 32% reduction of the AUC of imatinib. In addition, according to bioequivalence criteria the interactions in both imatinib trials were shown to be clinically relevant [11, 12].

The results with the anticancer drugs were in accordance with the clinical interaction studies with midazolam. Hence, midazolam has been shown to be a good predictor for CYP3A4-based interactions between SJW and anticancer drugs.

Milk thistle

Milk thistle (*Silybum marianum*) is the fourth most popular herbal medicine among cancer patients [6]. Milk thistle is supposed to have anti-oxidative, anti-inflammatory, anti-fibrotic, liver regenerating and anti-lipid-peroxidative effects. It is commonly used for gastrointestinal disturbances or diseases of the liver or biliary tract [48, 49]. Milk thistle contains silymarin, which is a mixture of at least seven flavonolignans and one flavonoid. The main constituent of silymarin is the flavonolignan silibinin [50].

In vitro effects of milk thistle on CYP3A4 and drug metabolism

To determine the effect of milk thistle on CYP3A4, several *in vitro* studies have been performed (Table 2A). The inhibitory effect of the milk thistle extract, silymarin and silibinin on CYP3A4 activity has been demonstrated in microsomes [51-55]. In hepatocytes silymarin also inhibited CYP3A4 activity [56]. Furthermore, inhibition of CYP3A4 activity by silibinin was demonstrated in *Escherichia Coli* MV1304 cells [57]. The extent of CYP3A4 inhibition by milk thistle, silymarin and silibinin (IC_{50} values of 27 - 60 µM [53-55]) is moderate to weak compared to ketoconazole (IC_{50} of 0.03 µM [38]). Furthermore, silibinin had no effect on CYP3A4 activity in baculosomes [37] and on CYP3A4

Table 2A. Effect of milk thistle on the activity of CYP3A4 *in vitro*

Models	CAM/component	Milk thistle product	Assay	Results	Ref
Human liver microsomes	MT extract (normalized to 10 µM silibinin)	Indena USA Inc., Seattle, USA	HPLC-UV (midazolam and testosterone)	43% ↓ CYP3A4 activity	51
Human liver microsomes	100 µM silymarin	Madaus GmbH, Cologne, Germany	LC-MS (testosterone)	> 90% ↓ CYP3A4 activity	52
Human liver microsomes	0-100 µM silibinin	Ivax-CR, Opava, Czech Republic	HPLC-UV (nifedipine)	↓ CYP3A4 activity IC_{50} = 27 & 60 µM	53
Human liver microsomes	0-300 µM silibinin	Purified and provided by Dept. of Chemistry, Madaus, Germany	HPLC-UV (nifedipine)	↓ CYP3A4 activity IC_{50} = 29 & 46 µM	55
Human liver microsomes	0-400 µM silibinin	Ivax-CR, Opava, Czech Republic	HPLC-UV (testosterone)	↓ CYP3A4 activity IC_{50} = 49.8 µM	54
Human hepatocytes	100 µM silymarin	Sigma, St. Louis, USA	HPLC-UV (testosterone)	50% ↓ CYP3A4 activity	56
Human hepatocytes	0-100 µM silibinin	Purified and provided by Galena Opava a.s., Czech Republic	Northern blotting	↔ mRNA, protein	58
E. coli MV1304 cells	0-250 µM silibinin	Sigma-Aldrich, St. Louis, USA	HPLC-UV (testosterone)	↓ CYP3A4 activity K_i = 132 µM	57
Baculosomes	0-50 µM silibinin	Sigma Chemical Co., St. Louis, USA	Fluorescence assay (Vivid red)	↔ CYP3A4 activity	37
Baculosomes	0-50 µM silibinin	Sigma Chemical Co., St. Louis, USA	HPLC-UV (cortisol)	↔ CYP3A4 activity	37
Caco-2 cells	0-75 µg/mL MT extracts	Local retail outlets, Ottawa, USA	RT-PCR and Western blotting	↓ mRNA, protein	59
Caco-2 cells	0-10 µM silibinin	Sigma-Aldrich, St. Louis, USA	RT-PCR and Western blotting	↓ mRNA ↑ protein	59

Abbreviations: RT-PCR = real-time polymerase chain reaction; HPLC-UV = high-performance liquid chromatography with ultraviolet detection; LC-MS = liquid chromatography mass spectrometry; BQ = 7-benzyloxyquinoline; BFC = 7-benzyloxy-4-trifluoromethylcoumarin; IC_{50} = half maximal inhibitory concentration; K_i = *in vitro* inhibition constant. ↔ = unchanged, ↓ = decrease and ↑ = increase.

mRNA and protein expression in hepatocytes [58], although sufficient concentrations (0 - 100 µM) of silibinin were examined. In Caco-2 cells (human adenocarcinoma colon cells), both milk thistle extract and silibinin inhibited CYP3A4 mRNA expression, but their effects on CYP3A4 protein expression were conflicting: milk thistle caused a decreased expression, while silibinin caused an increased expression of CYP3A4 [59]. Overall, the majority of *in vitro* studies showed inhibition of CYP3A4 by milk thistle. Based on these findings, it is interesting to investigate whether milk thistle also inhibits CYP3A4 in the clinic.

Clinical pharmacokinetic interactions between milk thistle and midazolam

Two one-sequence crossover studies have been performed with milk thistle and oral midazolam (Table 2B). In the first study with twelve healthy volunteers, a 28-day supplementation of 175 mg milk thistle twice daily (standardized to 80% silymarin) did not significantly affect the pre- and postsupplementation metabolic ratio of 1-hydroxymidazolam/midazolam [60]. Accordingly, in a later interaction study fourteen days of milk thistle intake (300 mg, three times daily, standardized to 80% silymarin) did not influence the AUC of midazolam or the metabolic ratio of 1-hydroxymidazolam/midazolam [61]. A lack of clinical CYP3A4 inhibition could be related to the low bioavailability of silymarin (including silibinin), which is the component responsible for CYP3A4 inhibition *in vitro* [60-62]. Silymarin has been shown to be practically insoluble in water, and in *in vitro* studies it was dissolved in the organic solvents dimethyl sulfoxide (DMSO) or methanol. Unfortunately, the bioavailability of silymarin was not measured in the two reported milk thistle-midazolam studies. The reported data indicate that commercially available milk thistle products, dosed as 175 – 300 mg two-three times daily, do not significantly alter clinical CYP3A4 activity. Thus, the observed inhibition of CYP3A4 by milk thistle *in vitro* could not be reproduced in clinical studies with midazolam.

Clinical pharmacokinetic interactions between milk thistle and anticancer drugs

One single-sequence crossover trial explored the pharmacokinetic interaction between milk thistle and irinotecan [47]. Short-term (four days) and long-term (twelve days) intake of 200 mg milk thistle extract (three times daily) did not significantly alter the pharmacokinetics of irinotecan (125 mg/m^2, IV) or its metabolites SN-38, SN-38G and APC (Table 2B). These results are in concordance with the results obtained with midazolam.

The two studies with midazolam and the one with irinotecan used different brands of milk thistle products, but all were standardized to 80% silymarin. The milk thistle dose varied between 175 – 300 mg, administered two or three times daily. Despite the reported wide variation in bioavailability and silymarin-release properties in different milk thistle products [61], the clinical outcome was the same. Presumably, the water solubility in the

gastrointestinal tract and bioavailability of silymarin was too low to achieve inhibition of intestinal and hepatic CYP3A4. This is supported by the study with irinotecan [47], in which analysis of silibinin plasma levels (C_{max} silibinin: range 0.0249 – 0.257 μM) revealed that these levels were too low to achieve CYP3A4 inhibition considering reported IC_{50} values of silibinin ranging from 27 to 60 μM [53-55].

The lack of an interaction between milk thistle and irinotecan could thus be predicted based on the results obtained with midazolam. As milk thistle is poorly bioavailable [63], it will mainly affect intestinal CYP3A4. Therefore, milk thistle is expected to have a greater effect on systemic exposure of oral midazolam than on exposure to IV drugs such as irinotecan.

Table 2B. Effect of milk thistle on the pharmacokinetics of selected substrate drugs in healthy volunteers and patients

Subjects	Dose milk thistle	Milk thistle product	Substrate	Results	Ref
Healthy volunteers (*n* = 12)	175 mg (80% silymarin), twice daily, 28 days	Wild Oats Markets, Inc, Boulder, USA	MDZ (P.O.)	No sign. effect on 1-OH MDZ / MDZ serum ratio	60
Healthy volunteers (*n* = 19)	300 mg (80% silymarin), thrice daily, 14 days	Enzymatic Therapy, Inc, Green Bay, USA	MDZ (P.O.)	No sign. effect on AUC0-∞ MDZ, and 1-OH MDZ / MDZ serum ratio	61
Cancer patients (*n* = 6)	200 mg (80% silymarin), thrice daily, 4 and 12 days	GNC, Pittsburgh, USA	Irinotecan (IV)	No sign. effect on AUC irinotecan, SN-38, SN-38G, APC.	47

Abbreviations: $AUC_{0-\infty}$ = area under the serum concentration-time curve from time zero extrapolated to infinity; no sign. = not statistically significant; 1-OH MDZ = 1-hydroxymidazolam; MDZ = midazolam; P.O. = oral administration; IV = intravenous administration.

Garlic

Garlic (*Allium sativum*) is listed in the top 10 of CAM used by cancer patients [6]. This herb is assumed to have anticancer, immune-enhancing, antiplatelet, chemopreventive, antihypertensive, procirculatory, hypolipidemic and hepatoprotective effects [64, 65]. One of the major garlic components responsible for these effects is allicin [64]. After crushing garlic or wetting garlic powder, allicin is formed from the garlic constituent alliin by the enzyme aliinase [66].

In vitro effects of garlic on CYP3A4 and drug metabolism

Several *in vitro* studies have been performed to determine whether garlic and its major component allicin are able to affect CYP3A4. In two *in vitro* studies, garlic extracts did not inhibit CYP3A4 activity in Supersomes™ [67] and in Fa2N-4 cells [68]. Furthermore, the garlic extract had no effect on CYP3A4 transcription in HepG2 cells (Table 3A) [69]. However, in the same study the garlic extract increased mRNA expression which was not likely to be PXR-mediated. In

contrast, allicin [65] and the extracts of commercial garlic products [64] were also shown to inhibit CYP3A4 activity in Supersomes™ [64, 65]. However, the decreased fluorescent signal is not likely to be caused by inhibition of CYP3A4 by garlic, since very high concentrations of garlic (25 mg/mL) were used compared to the used concentrations of garlic (0 – 200 µg/mL) in the studies demonstrating no effect. More likely, the great amount of garlic extract interfered with the fluorescent signal (i.e. quenching). Since the majority of the *in vitro* studies demonstrated no effect, it is possible that garlic has also no inhibitory effect on CYP3A4 in the clinic.

Table 3A. Effect of garlic on the activity of CYP3A4 *in vitro*

Models	CAM/component	Garlic product	Assay	Results	Ref
Supersomes™	0-98 µM allicin	LKT Laboratories, St. Paul, USA	Fluorescence assay (BFC)	↓ CYP3A4 activity IC_{50} = 43.73 µM	65
Supersomes™	0-98 µM allicin	LKT Laboratories, St. Paul, USA	Fluorescence assay (BzRes)	↓ CYP3A4 activity IC_{50} = 60.10 µM	65
Supersomes™	25 mg/mL garlic extracts	Local commercial outlets, Ottawa, USA	Fluorescence assay (BzRes)	17.4 – 95.8% ↓ CYP3A4 activity	64
Supersomes™	0-5 ng/mL garlic	Local pharmacy, Bremen, Germany	HPCL-UV (testosterone)	↔ CYP3A4 activity	67
Fa2N-4 cells	0-200 µg/mL garlic extract	Nature's Way, Springville, USA	RT-PCR LC-MS (midazolam)	↔ mRNA and CYP3A4 activity	68
Human hepatocytes	0.1 µg/mL garlic	Local commercial outlets, Ottawa, USA	Northern blotting	2 times ↑ mRNA	69
HepG2 cells	0.1 µg/mL garlic	Local commercial outlets, Ottawa, USA	Reporter gene assay	no fold induction of CYP3A4	69

Abbreviations: BFC = 7-benzyloxy-4-trifluoromethylcoumarin; BzRes = 7-benzyloxyresorufin; HPLC-UV = high-performance liquid chromatography with ultraviolet detection; LC-MS = liquid chromatography mass spectrometry; RT-PCR = real-time polymerase chain reaction; IC_{50} = half maximal inhibitory concentration. ↑ = increase; ↓ = decrease; ↔ = unchanged.

Clinical pharmacokinetic interactions between garlic and anticancer drugs

Based on the absence of a clinical pharmacokinetic interaction between garlic and midazolam, interactions with anticancer drugs predominantly metabolized by CYP3A4 are not expected. Indeed, in a one-sequence crossover study with breast cancer patients supplementation of 600-mg enteric coated garlic tablets (3,600 µg allicin per tablet) twice daily for twelve days did not significantly alter the pharmacokinetics of docetaxel (Table 3B) [71], which is mainly metabolized by CYP3A4 into four major metabolites [72]. Despite minimizing gastric inactivation of alliinase by using enteric coated tablets, no interaction occurred. This result was in concordance with the midazolam studies. Thus, the clinical use of midazolam has proven to be a good predictor of pharmacokinetic interactions with other CYP3A4 substrates, such as docetaxel.

Table 3B. Effect of garlic on the pharmacokinetics of selected substrate drugs in healthy volunteers and patients

Subjects	Dose garlic	Garlic product	Substrate	Results	Ref
Healthy volunteers (*n* = 12)	500 mg garlic oil, thrice daily, 28 days	Wild Oats Markets, Inc, Boulder, USA	MDZ (P.O.)	No sign. effect on 1-OH MDZ / MDZ serum ratio	31
Healthy volunteers (*n* = 12)	500 mg garlic oil, thrice daily, 28 days	Vitamer, Lake Forest, USA	MDZ (P.O.)	No sign. effect on 1-OH MDZ / MDZ serum ratio	30
Breast cancer patients (*n* = 10)	600 mg (3,600 µg allicin), twice daily, 12 days	GarliPure, Maximum Allicin Formula, Natrol, Chatsworth, USA	Docetaxel (IV)	No sign. effect on AUC docetaxel	71

Abbreviations: AUC_{0-8} = area under the plasma concentration-time curve from 0-8 h; no sign. = not statistically significant; 1-OH MDZ = 1-hydroxymidazolam; MDZ = midazolam; P.O. = oral administration; IV = intravenous administration; PK = pharmacokinetics.

P. ginseng

P. ginseng belongs to the twenty most frequently used CAM among cancer patients [6]. *P. ginseng* has been supposed to have anti-neoplastic, anti-aging, anti-oxidative and immunomodulatory effects [73, 74]. This herb is used for cancer prevention, erectile dysfunction, improved cognitive functions and enhanced physical functions [75]. *P. ginseng* consists of ginsenosides and sapogenins, of which ginsenosides are the main components [73].

In vitro effects of *P. ginseng* on CYP3A4 and drug metabolism

The effect of *P. ginseng* on CYP3A4 has been determined in several *in vitro* studies (Table 4A). The flavonoid kaempferol was shown to inhibit CYP3A4 activity in baculosomes and microsomes [37]. Ginsenosides Rh_2, C-K and all sapogenins exhibited moderate CYP3A4 inhibition in baculosomes and Rg_3 showed relatively weak CYP3A4 inhibition [73]. In microsomes, ginsenosides Rh_1 and F_1 inhibited CYP3A4 activity respectively by 54% and 60% [51, 74]. Ginsenoside Rd [76, 77] and sapogenins PPD and PPT [76] were shown to be relatively moderate and strong inhibitors of CYP3A4 activity in microsomes with corresponding IC_{50} values of respectively 81.7, 14.1 and 7.1 µM. Ginsenosides Rb_2 and Rc, however, had limited inhibitory effects in these microsomes. Also the inhibitory effect of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg was weak compared to ketoconazole [38]. Long-term (48 h) exposure of *P. ginseng* had no effect on CYP3A4 mRNA expression in hepatocytes and CYP3A4 transcription in HepG2 cells [69]. Thus, *P. ginseng* is apparently no inducer of CYP3A4. Overall, the majority of *in vitro* studies showed that *P. ginseng* is a competitive inhibitor of CYP3A4. Therefore, an effect of *P. ginseng* on CYP3A4 could be expected in the clinic.

Table 4A. Effect of *P. ginseng* on the activity of CYP3A4 *in vitro*

Models	CAM/component	*P. ginseng* product	Assay	Results	Ref
Human hepatocytes	500 µg/mL ginseng	Local commercial outlets, Ottawa, USA	Northern blotting	↔ mRNA	69
HepG2 cells	500 µg/mL ginseng	Local commercial outlets, Ottawa, USA	Reporter gene assay	no fold induction of CYP3A4	69
Baculosomes	0-50 µM kaempherol	Sigma Chemical Co., St. Louis, USA	fluorescence assay (Vivid red)	↓ CYP3A4 activity IC_{50} = 260.7 nM	37
Baculosomes	0-50 µM Rh_2, C-K[e] and Rg_3	Obtained and purified from P. ginseng fruits, Liaoning Xinbin Pharmaceutical, Fushun, China	fluorescence assay (Vivid red)	↓ CYP3A4 activity IC_{50} = resp. 9.8; 9.1; 25.2 µM	73
Rat liver microsomes	50 µM kaempherol	Sigma Chemical Co., St. Louis, USA	HPLC-UV (cortisol)	89.7% ↓ CYP3A4 activity	37
HLM	0-100 µM Rh_1, F_1	Fermenta Herb Institute Inc., Tokyo, Japan	HPLC-UV (testosterone)	↓ CYP3A4 activity IC_{50} = resp. 76.9; >100 µM	74
HLM	10 µM Rh1, F1	Flachsmann Canada Ltd., Brampton, Canada	HPLC-UV (midazolam)	resp. 54% and 60% ↓ CYP3A4 activity	51
HLM	0-100 µM PPD, PPT, Rd	Fermenta Herb Institute Inc., Tokyo, Japan	HPLC-UV (testosterone)	↓ CYP3A4 activity IC_{50} = resp. 14.1; 7.1; 81.7 µM	76
HLM	0-200 µM Rd, Rb_2, Rc	Indofine Chemical Co., Somerville, USA	HPLC-UV (testosterone)	↓ CYP3A4 activity IC_{50} = resp. 62, 133, 197 µM	77
HLM	0-200 µM Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg	Indofine Chemical Co., Somerville, USA	Fluorescence assay (BzRes and BFC)	All IC_{50} > 200 µM IC_{50}'s of Rd resp. 74, 58 µM and of Rb_2 resp. 178, 165 µM	38

Abbreviations: HLM = human liver microsomes; HPLC-UV = high-performance liquid chromatography with ultraviolet detection; IC_{50} = half maximal inhibitory concentration. ↓ = decrease; ↔ = unchanged.

Clinical pharmacokinetic interactions between *P. ginseng* and midazolam

In contrast to the results obtained *in vitro*, inhibition of CYP3A4 by *P. ginseng* has not been reported in clinical studies with midazolam (Table 4B). The sequence of *P. ginseng* and midazolam administration was fixed in these studies. In twelve healthy volunteers a 28-day supplementation period of 500 mg *P. ginseng* (three times daily, standardized to 5% ginsenosides) did not significantly affect the 1-hydroxymidazolam/midazolam serum ratio [31]. Also in elderly volunteers, no significant effect of *P. ginseng* (500 mg, three times daily, standardized to 5% ginsenosides) on the 1-hydroxymidazolam/midazolam serum ratio was observed [30]. Presumably, the inhibiting potency of ginsenosides is too low to cause significant clinical CYP3A4 inhibition. Rather high plasma concentrations of ginsenosides are required for inhibition of CYP3A4, as the IC_{50} values of several ginsenosides have shown to be more than 1,000 times higher than the IC_{50} value of the potent CYP3A4 inhibitor ketoconazole [38].

One clinical study, however, found significant induction of CYP3A4 by *P. ginseng*. Intake of 500 mg *P. ginseng* (twice daily for 28 days, standardized to 5% ginsenosides) by healthy volunteers significantly decreased the AUC of oral midazolam by 34%, which suggested induction of CYP3A4 [78]. Interestingly, this significant result differed from the observations in the two other trials with midazolam, despite a similar composition of the *P. ginseng* product (500 mg extract, standardized to 5% ginsenosides) and a lower dosing frequency. Possibly, differences in assessments of CYP3A4 activity were responsible for these conflicting results. A one-hour postdose serum concentration ratio of 1-hydroxymidazolam/midazolam was the primary outcome in studies showing no apparent interaction between *P. ginseng* and midazolam [30, 31], while midazolam $AUC_{0-\infty}$ was calculated in the clinical study which suggested CYP3A4 induction by *P. ginseng* [78]. It has been reported that determination of the AUC of midazolam is more accurate to assess CYP3A4 activity than a single sample collection of midazolam [79]. Therefore it is possible that a CYP3A4 induction by *P. ginseng* may have been missed in the studies with a one-sampling strategy.

The discussed clinical studies with *P. ginseng* and midazolam show inconsistent results: two studies reported no pharmacokinetic interaction, while in one study the AUC of midazolam was significantly decreased after *P. ginseng* administration. The latter result indicates that *P. ginseng* could also reduce the systemic exposure of oncolytic substrates for CYP3A4.

Table 4B. Effect of *P. ginseng* on the pharmacokinetics and -dynamics of selected substrate drugs in healthy volunteers and patients

Subjects	Dose *P. ginseng*	*P. ginseng* product	Substrate	Results	Ref
Healthy volunteers (n = 12)	500 mg (5% ginsenosides), thrice daily, 28 days	Wild Oats Markets, Inc, Boulder, USA	MDZ (P.O.)	No sign. effect on 1-OH MDZ / MDZ serum ratio	31
Healthy volunteers (n = 12)	500 mg (5% ginsenosides), thrice daily, 28 days	Vitamer, Lake Forest, USA	MDZ (P.O.)	No sign. effect on 1-OH MDZ / MDZ serum ratio	30
Healthy volunteers (n = 12)	500 mg (5% ginsenosides), thrice daily, 28 days	Vitamer, Lake Forest, USA	MDZ (P.O.)	Significant 34% ↓ of $AUC_{0-\infty}$ MDZ	78
CML patient (n = 1)	Energy drink, daily intake, 3 months	Full Throttle energy drink	Imatinib (P.O.)	Imatinib-induced hepatotoxicity	80

Abbreviations: CML = chronic myelogenous leukemia; 1-OH MDZ = 1-hydroxymidazolam; MDZ = midazolam; No sign. = not statistically significant; P.O. = oral administration. ↓ = decrease.

Clinical pharmacokinetic interactions between *P. ginseng* and anticancer drugs

Thus far, no clinical interaction studies with *P. ginseng* and anticancer drugs have been reported. Only one case report described the onset of imatinib-induced hepatoxicity in a patient with chronic myelogenous leukemia (CML) who had been receiving imatinib for nine years. After concomitant use of imatinib and an energy drink containing *P. ginseng* for three months hepatotoxicity became apparent by abnormal liver function

test results (alanine aminotransferase 1069 U/L, aspartate aminotransferase 481 U/L, alkaline phosphatase 124 IU/L, total bilirubin 1.4 mg/dL, albumin 4.0 g/dL) and liver biopsy [80]. Liver enzyme levels returned to normal after discontinuation of imatinib and *P. ginseng* intake and treatment with prednisone. Restart with imatinib did not result in recurrent elevations in liver enzyme levels. Imatinib itself may cause hepatoxicity, but this usually occurs within the first 1-2 years of therapy. It is more likely that the described hepatoxicity was caused by concomitant administration of *P. ginseng*, as the Horn drug interaction probability scale suggested a probable interaction between imatinib and *P. ginseng*. According to the authors the underlying mechanism of this interaction might be inhibition of CYP3A4 and possibly also the drug transporter P-gp by *P. ginseng* (Table 4B). However, the proposed pharmacokinetic interaction between *P. ginseng* and imatinib in the case report [80] should be questioned, as no pharmacokinetic parameters of imatinib or its metabolites have been determined in the affected patient. Furthermore, the ingested energy drink also contained other components besides *P. ginseng* (e.g. guarana extract, caffeine, taurine and B-vitamins) [81] which might have affected the metabolism of imatinib. Thus, to draw more definitive conclusions about a pharmacokinetic interaction between *P. ginseng* and imatinib, a (pre)clinical interaction study with an appropriate sample size and pharmacokinetic endpoints should be executed.

DISCUSSION

The review of available literature indicates that the value of *in vitro* research to predict clinical effects on CYP3A4 is different for each reported herb. The *in vitro* inductive effect of SJW was extrapolatable to the clinic, using midazolam and the anticancer drugs irinotecan and imatinib as CYP3A4 substrates (Table 1B). Also for garlic *in vitro* results were extrapolatable: no effect on CYP3A4 has been observed both in *in vitro* and in clinical studies (Tables 3A and 3B). However, for milk thistle and *P. ginseng* CYP3A4 inhibition was observed *in vitro*, but these results were not reproducible in clinical studies (Tables 2B and 4B).

The poor correlation between *in vitro* and clinical results observed for milk thistle and *P. ginseng* reveal limitations that are commonly observed for CAM-drug interaction studies. For example, the phytochemical content (and the interacting potential) of herbal medicines is usually highly variable among different brands, but also within batches [82]. This has been demonstrated for 25 different ginseng preparations for which phytochemical analysis showed a high interproduct variability in ginsenosides content (15- to 36-fold variation) [83]. Furthermore, measured concentrations of ginsenosides were lower than the labeled amount of ginsenosides in the majority of *P. ginseng* preparations. In the described clinical *P. ginseng* studies, however, phytochemical analysis of the *P. ginseng* products were

in agreement with the amount of ginsenosides stated on the label. Possibly, ginsenosides are too weak CYP3A4 inhibitors (as shown in *in vitro* studies) to achieve clinically significant interactions.

Besides variations in phytochemical content, also variability in drug-releasing properties and bioavailability are observed in commercial CAM products. Analysis of nine milk thistle products showed that the amount of released silibinin in an aqueous buffered solution varies considerably, ranging from 0 to 85% [84]. Furthermore, bioavailability of milk thistle has shown to be poor and highly variable. It is likely that these factors all contribute to a lack of clinically significant effects of milk thistle on CYP3A4.

To improve extrapolatability of *in vitro* interaction studies it is recommended to confirm effects of CAM in multiple test systems and assays for quantification within a single *in vitro* study. By this, more robust results can be obtained and it is also possible to prevent interlaboratory differences and the use of different product brands. The extrapolatability can also be improved by assays that closely mimic physiological conditions in terms of pH and the composition of gastric fluid. Early detection of poor soluble components will then reveal the low likelihood of CYP3A4-mediated CAM-drug interactions in humans.

In contrast to the occasionally poor *in vitro* – clinical extrapolation of CYP3A4-based interactions, the clinical use of midazolam has shown to be a good predictor of pharmacokinetic interactions between CAM and anticancer drugs metabolized by CYP3A4. Specifically, the occurrence of clinical CYP3A4-mediated interactions between anticancer drugs and SJW, milk thistle and garlic, correlated well with clinical results obtained with these CAM and midazolam.

Recommendations

Based on the available pharmacokinetic data of SJW, milk thistle, garlic and *P. ginseng*, it is possible to provide recommendations towards concomitant use of these CAM by cancer patients.

For SJW it is recommended not to combine hyperforin-rich SJW extracts with imatinib or irinotecan in order to prevent undertreatment of cancer patients. Additionally, caution is warranted when combining SJW with other anticancer drugs metabolized by CYP3A4 (e.g. docetaxel, paclitaxel, vinorelbine, gefitinib), as the systemic exposure and possibly also the efficacy of these therapies could be diminished. However, it is presumably safe to combine hyperforin-poor (≤ 0.13 mg hyperforin per day) SJW extracts with imatinib or irinotecan as this hyperforin dose did not significantly affect the systemic exposure to midazolam [45].

For milk thistle and garlic it is presumably safe to combine these CAM with respectively irinotecan and docetaxel and other anticancer drugs metabolized by CYP3A4.

For *P. ginseng* it is not possible to provide recommendations about the concomitant use with anticancer drugs, because clinical interaction studies should be executed with *P. ginseng* and anticancer drugs metabolized by CYP3A4. However, it should be noted that

the determined absence or presence of pharmacokinetic interactions between CAM and anticancer drugs are only applicable for the tested brands of CAM products. Due to substantial interproduct variabilities, the interacting potential of other brands may differ.

CONCLUSION

This review summarizes that *in vitro* data are used to discover effects of CAM on CYP3A4 and for certain CAM, such as SJW, these effects can be extrapolated to clinical studies. Occasionally, significant effects on CYP3A4 *in vitro* do not predict significant interactions in clinical trials. These discrepancies can be largely attributed to differences between *in vitro* test systems and the physiological environment. To improve extrapolation from *in vitro* to the clinic, *in vitro* conditions should closely mimic physiological conditions, as accurate prediction of clinical pharmacokinetic interactions is complicated by several factors (e.g. poor pharmaceutical availability, solubility and bioavailability of CAM). Therefore, clinical studies are required for confirmation and assessment of the clinical relevance of herb-drug interactions obtained *in vitro*. In clinical trials midazolam has been shown to be a useful probe drug to correctly predict the presence or absence of clinical CYP3A4-based interactions between CAM and anticancer drugs.

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CHAPTER 2.2

Letter to the Editor regarding "A Prospective, Controlled Study of the Botanical Compound Mixture LCS101 for Chemotherapy-Induced Hematological Complications in Breast Cancer" by Yaal-Hahoshen et al. (The Oncologist 2011;16:1197-1202).

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Based on the results of a prospective, randomized, placebo-controlled study of Yaal-Hahoshen *et al*. [1], the authors concluded that the botanical mixture LCS101 prevented hematological complications in breast cancer patients undergoing anthracycline- and taxane-based chemotherapy. They also concluded that the addition of LCS101 to conventional chemotherapy regimens "is both safe and feasible in patients with early breast cancer, …". However, we believe that these conclusions should be interpreted with caution because the present study has a few major limitations. First, the possible pharmacodynamic (PD) effect of LCS101 on the bone marrow was not evaluated directly in the present study. Yaal-Hahoshen *et al*. [1] primarily determined hematological parameters by counting erythrocytes, leukocytes, neutrophils, lymphocytes, and thrombocytes in the peripheral blood. These parameters only secondarily reflect bone marrow function. It would be more accurate to evaluate the growth of progenitor cells such as colony-forming unit (CFU)-granulocytes, erythroids, macrophages and megakaryocytes; burst-forming units-erythroids; and CFU-granulocyte-macrophages [2].
Currently, the effect of LCS101 on hematopoietic function has barely been investigated. The authors only reported that the "addition of LSC101 to doxorubicin led to significantly better peripheral neutrophil counts, and preserved splenic erythrocyte and leukocyte counts" in a mouse breast cancer model. Unfortunately, these data were not published.
For a few individual LCS101 components, however, a PD effect on bone marrow function could be expected. For example, the production of erythroid progenitor cells has been stimulated by *Ophiopogon japonicus* in mice [3], and in patients with chronic aplastic anemia hematopoietic recovery has been promoted by *Astralagus membranaceus* [4]. As Yaal-Hahoshen *et al*. [1] reported, it is still unclear what the implications of these activities are for chemotherapy-induced hematological toxicity. Additionally, it is unknown whether the components may interact synergistically or additively with each other, which complicates predicting the effect of LCS101 on hematopoietic function.
Second, in the present study no pharmacokinetic (PK) analysis of the administered anticancer drugs (such as doxorubicin, paclitaxel, and docetaxel) was performed. Therefore, it is not possible to exclude possible PK interactions between LCS101 and these anticancer drugs. Regarding the effects of LCS101 on the PK of anticancer drugs, there is no information available because no PK interaction studies with LCS101 have been performed.
However, based on *in vitro* results with individual LCS101 components, PK interactions between LCS101 and anticancer drugs metabolized by cytochrome P450 (CYP) 3A4 cannot be ruled out. Using a reporter gene assay and real-time polymerase chain reaction, six LCS101 components induced pregnane X receptor (PXR)-regulated CYP3A4 transcription in HepG2 cells: *Astragalus membranaceus*, *Poriae cocos*, *Atractylodes macrocephala*, *Lycium chinense*, *Ophiopogon japonicus*, and *Paeonia lactiflora* [5]. Furthermore, according to our preliminary results based on a reporter gene assay, the LCS101 compound *Oldenlandia*

diffusa is also a potent inducer of PXR-regulated CYP3A4 transcription in LS180 cells (unpublished data). Thus, because CYP3A4 is involved in the metabolism of doxorubicin [6], docetaxel [7], and paclitaxel [8], CYP3A4 induction might lead to lower systemic exposure of these anticancer drugs and consequently to the reported lower incidence of hematological toxicities in cancer patients who received LCS101. Therefore, it is important to evaluate the potential PK interactions between LCS101 and anticancer drugs in clinical PK studies.
Third, the PD effect of LCS101 on the tumor, and thus the efficacy of the administered anticancer drugs, was not evaluated. The statement from Yaal-Hahoshen *et al.* [1] that the addition of LCS101 to conventional chemotherapy is safe and feasible implies that the antitumor effect of chemotherapy is not negatively affected by LCS101. However, their study did not evaluate the possible PD effects of LCS101 on the tumor. This would have provided valuable information because there currently are no clinical data regarding the effects of LCS101 on the antitumor effect of the administered anticancer drugs. It has only been shown for one individual LCS101 component (*Ligustrum lucidum*) that doxorubicin-induced apoptosis was enhanced in human colorectal carcinoma DLD-I cells [9]. Determination of the PD effects of LCS101 on the tumor could have been assessed by tumor size evaluation using the Response Evaluation Criteria in Solid Tumors. In the present study, however, the sample size was too small to exclude a PD effect of LCS101 on the tumor. To assess the PD effect of LCS101 on the tumor during neoadjuvant chemotherapy using a noninferior study design, ~ 1,200 patients would be required based on a significance level of 0.05, probability (power) of 0.80, noninferiority margin of 5%, and overall clinical response rate of 86% for chemotherapy with doxorubicin plus cyclophosphamide [10]. This is a substantially larger number of patients than the 18 patients who underwent neoadjuvant chemotherapy in the present study.
In conclusion, Yaal-Hahoshen *et al.* [1] claimed that the addition of LCS101 to conventional chemotherapy is safe and feasible in patients with early breast cancer. However, their study lacks essential information regarding the hematoprotective mechanism of action of LCS101, potential PK interactions with chemotherapy, and the PD effects of LCS101 on the tumor. Therefore, we recommend the execution of PK interaction studies with LCS101 and anticancer drugs. In addition, the PD effects of LCS101 on hematopoietic function and on tumors should also be evaluated clinically. Until these studies have been executed, we advise not to combine LCS101 with anticancer drugs.

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CHAPTER 3

The effect of *Echinacea purpurea* on the pharmacokinetics of docetaxel in cancer patients: CYP3A4 induction

CHAPTER 3.1

A sensitive LC-MS/MS method for the quantitative analysis of the *Echinacea purpurea* constituent undeca-2-ene-8,10-diynoic acid isobutylamide in human plasma

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ABSTRACT

Background: *Echinacea purpurea* is one of the most popular herbal medicines and is known for its immunostimulatory effects. Alkylamides are the main lipophilic components of *E. purpurea* that contribute to its pharmacological actions. For quantification in human plasma of one of these alkylamides, undeca-2-ene-8,10-diynoic acid isobutylamide, a sensitive LC-MS/MS assay has been developed and validated.

Materials and Methods: Plasma samples were pretreated using liquid-liquid extraction with a mixture of diethyl ether and n-hexane (50:50, v/v). Dried extracts were reconstituted in 50 µL of acetonitrile-water (50:50, v/v) after which 15 µL of sample was injected into the HPLC system. HPLC was performed using a Polaris 3 C18-A column (50 mm x 2 mm ID) and isocratic elution with acetonitrile-water (50:50, v/v) containing 0.1% formic acid at a flow rate of 0.3 mL/min. Subsequently, electrospray ionization in the positive ion mode followed by tandem mass spectrometry was performed for detection. The total run time was 3 min.

Results and Conclusions: The assay was validated over a concentration range from 0.05 to 50 ng/mL for undeca-2-ene-8,10-diynoic acid isobutylamide, with 0.05 ng/mL being the lower limit of quantification using 1.0 mL plasma samples. Inter-assay inaccuracy (± 12.7%), within-day and between-day precisions (CV ≤ 8.23%) were acceptable. Further, undeca-2-ene-8,10-diynoic acid isobutylamide was found to be chemically stable under relevant conditions. Finally, the applicability of this assay has been successfully demonstrated in a pharmacokinetic experiment in which a human volunteer ingested a commercial extract of *E. purpurea*.

INTRODUCTION

Echinacea purpurea is one of the most popular herbal medicines, and is widely used because of its supposed beneficial effects on the immune system. Examples of reported indications are the common cold, flu and upper respiratory infections. The active ingredients considered responsible for the immunostimulatory and anti-inflammatory actions are caffeic acid derivatives, polysaccharides, glycoproteins and alkylamides [1]. Alkylamides are the main lipophilic constituents and so far 17 structures have been identified [2]. One of the alkylamides is undeca-2-ene-8,10-diynoic acid isobutylamide (UDAI, Figure 1A), involved in the anti-inflammatory activity of *E. purpurea*. In combination with other *Echinacea* alkylamides, this compound was found to significantly inhibit TNF-α and nitric oxide production in mouse macrophage cells [3]. Further proof of the anti-inflammatory effects of UDAI was given by Hinz *et al.*, who demonstrated that UDAI was able to inhibit COX-2-dependent prostaglandin E_2 formation [4]. Inhibition of COX-2 has been proven to effectively suppress pain and inflammation. In addition, UDAI was also reported to inhibit T-cell IL-2 inhibition [5]. In our clinic, the potential pharmacokinetic interaction between *E. purpurea* extract and an anticancer agent will be studied in patients. To check the compliance of *E. purpurea* intake, the *Echinacea* alkylamide UDAI was selected to be monitored in plasma collected from these patients. For this purpose, a bioanalytical assay has to be developed and validated.

Until now, few bioanalytical assays for the quantification of alkylamides in biological matrix (plasma, serum) have been developed. In one paper [6] a method was described for the quantification of eight alkylamides in human plasma using liquid chromatography coupled with mass spectrometry (LC-MS). Solid-phase extraction (SPE) was used for sample pretreatment. Drawbacks of this method were the large amount of solvents for SPE and a run time of 23 min. Furthermore, this method was executed in the single ion monitoring (SIM, LC-MS) mode, which is not as specific as *tandem* mass spectrometry (LC-MS/MS).

Another research group has also developed LC-MS/MS methods for the bioanalysis of alkylamides in human serum and plasma [7-9]. These methods were used for evaluation of the pharmacokinetics of alkylamides after ingestion of *Echinacea* preparations by human volunteers. All plasma and serum samples were pretreated using SPE before performing the LC-MS/MS analysis. Disadvantages of these LC-MS/MS methods were, however, a long run time of 20 min per sample [7-9] and the large solvent and sample volumes used (14 mL serum [8] and 16 mL plasma [9]).

The previously described LC-MS/MS assay for UDAI, has been used to study the pharmacokinetics of UDAI in volunteers [7]. In that study, 2.5 mL of an 60% ethanolic extract from the roots of *Echinacea angustifolia* was administered to 11 volunteers. The mean maximum plasma concentration of 1.87 ng/mL UDAI was found. The lower

limit of quantification (LLQ) of this assay was 0.08 ng/mL. In our clinical study, 20 drops (approximately 1 mL) of an 65% ethanolic extract of *E. purpurea* will be administered. Compared to the pharmacokinetic study, a mean maximum plasma concentration lower than 1.87 ng/mL was expected due to a 2.5 times lower volume of the ingested dose. Furthermore, UDAI is more abundant in *E. angustifolia* than in *E. purpurea* [2]. Therefore, we hypothesized that a LLQ of 0.08 ng/mL may not be sufficient for our clinical study. Furthermore, for practical reasons a shorter run time and a smaller sample volume than the previously developed bioanalytical assays for UDAI [6-9] was desirable. Therefore, we have developed a fast and sensitive LC-MS/MS method for the quantification of UDAI in human plasma. In the present assay, liquid-liquid extraction (LLE) was used for sample preparation. As no isotopically labeled internal standard was commercially available, benzanilide was used as internal standard which was also used for this purpose in previous studies (Figure 1B) [7-10]. Further, the presently described assay has been fully validated according to the FDA guidelines on Bioanalytical Method Validation [11].

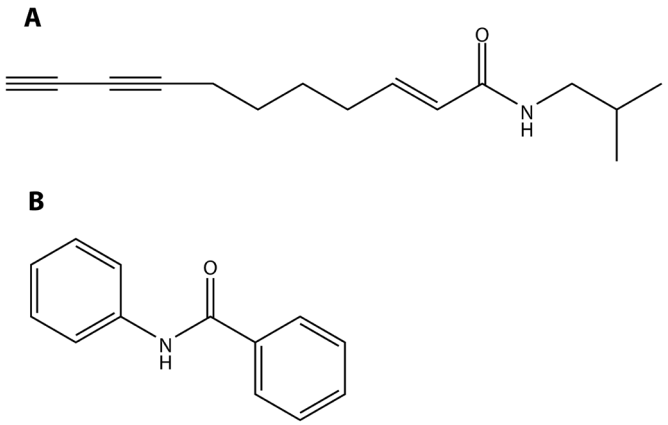

Figure 1. Chemical structures of (A) undeca-2-ene-8,10-diynoic acid isobutylamide and (B) benzanilide.

MATERIALS AND METHODS

Reagents and chemicals

UDAI ($C_{15}H_{21}NO$) was purchased from ChromaDex, Inc (Irvine, CA, USA). Benzanilide ($C_{13}H_{11}NO$) originated from Acros Organics (Leicestershire, UK). LC-MS grade water, ethanol and methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC grade n-hexane and analytical grade diethyl ether stabilized with 2,6-di-tert-butyl-4-methylphenol (BHT) and formic acid were purchased from Merck (Darmstadt, Germany). Blank, drug-free human plasma, containing heparin (Li) as anti-coagulant, was obtained from Innovative Research Inc. (Novi, MI, USA).

Instrumentation

The LC-MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-µ pumps (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with electrospray ionization (ESI). For data acquisition and processing, Xcalibur software (version 1.4, Thermo Fisher Scientific) was used.

Chromatographic conditions

Sample injections (15 µL) were made on a Polaris 3 C18-A column (50 mm x 2 mm ID, particle size 3 µm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 mm x 2 mm ID, particle size 3 µm, Varian). The column temperature was maintained at 40°C and the autosampler was set at 15°C. The total run time was 3 min. The mobile phase consisted of acetonitrile-water (50:50, v/v) containing 0.1% formic acid, which was delivered at a flow rate of 0.3 mL/min.

Mass spectrometry

The mass spectrometer operated in the positive ion mode with both quadrupoles set at 0.7 full width at half height (FWHM, unit resolution) and with dwell times of 200 ms. For UDAI, the mass transitions from *m/z* 232 to 105 were optimized and for benzanilide, responses from *m/z* 198 to 105 were monitored. The optimized collision energies were -16 V for UDAI and -20 V for benzanilide. For both compounds, the tube lens voltage was set at 101 V. Furthermore, spray voltage was set at 4500 V with an ion tube temperature of 210°C. Nitrogen sheath, ion sweep and auxiliary gasses were set at 49, 2.0 and 14 arbitrary units, respectively. Finally, the up-front collision-induced dissociation (CID) was set off and collision gas pressure (Ar) was set at 2.0 mTorr.

The robustness of the LC-MS/MS method was tested by monitoring the absolute area of UDAI and the ratio with the internal standard for two calibration samples at the concentration level of 5 ng/mL UDAI. One of these calibration samples was injected at the beginning of a run containing 55 plasma samples in total, whereas the other calibration sample was injected at the end of the same run. Relative standard deviations of the absolute signal of UDAI and the ratio were calculated.

Preparation of stock and working solutions

Two stock solutions of UDAI (0.1 mg/mL) from two independent weightings were prepared in methanol. One stock solution was used for the preparation of calibration standards, and the other solution was used to prepare quality control (QC) samples. For the calibration standards the stock solution was further diluted with control human heparinized plasma to obtain working solutions in a range from 2 to 10,000 ng/mL. The stock solution for QC samples was diluted with control human plasma to obtain a working solution of 10,000

ng/mL.
For the internal standard, a stock solution of benzanilide in methanol (1 mg/mL) was further diluted with reconstitution solvent (acetonitrile-water (50:50, v/v)) to obtain working solutions of 200 and 10,000 ng/mL.
The stock solutions and the internal standard working solutions were stored at -30°C until use, while the working solutions in plasma were used immediately after preparation.

Preparation of calibration standards and QC samples in plasma

Calibration standards were prepared freshly by diluting plasma working solutions with human plasma to obtain concentrations of 0.05, 0.1, 1, 5, 25 and 50 ng/mL UDAI. These standards were prepared and analyzed in duplicate.
QC samples were prepared in batches by diluting the QC working solution in volumetric flasks, obtaining QC samples at concentrations of 0.05 (LLQ), 0.15 (low), 5 (mid), 40 (high) and 50 (upper limit of quantification: ULQ) ng/mL UDAI. As no plasma concentrations of UDAI higher than 50 ng/mL were expected in patient samples, no dilution test sample above the ULQ (> ULQ sample) was prepared. QC samples were stored at -30°C until analysis.

Sample preparation

To 1 mL plasma samples 50 µL of the internal standard benzanilide (200 ng/mL) was added in a conical glass tube. After vortex-mixing, LLE was performed with 8 mL diethyl ether/n-hexane (50:50, v/v). Subsequently, the samples were shaken with a rotary-mixer for 10 min at 50 rpm. After centrifugation for 10 min at 3200 x *g* (4°C), the samples were stored at -30°C for 60 min. Next, the organic layer was decanted into another conical glass tube and evaporized under a stream of nitrogen at 40°C. The residue was reconstituted in 50 µL of acetonitrile-water (50:50, v/v) and vortex-mixed for approximately 15 s. After transferring the solution into 1.5 mL Eppendorf tubes the solution was centrifuged for 10 min at 13,500 rpm. Finally, the clear supernatant was transferred into a 250 µL glass insert placed in an autosampler vial.

VALIDATION PROCEDURES

A full validation of the assay in human heparinized plasma was performed according to the current FDA guidelines on Bioanalytical Method Validation [11].

Linearity

Six non-zero calibration standards at concentrations of 0.05, 0.1, 1, 5, 25 and 50 ng/mL UDAI were prepared freshly in duplicate for each run and analyzed in three separate runs.

Calibration curves (ratio of the areas of the analyte and internal standard peaks *versus* the nominal concentration) were fitted by least-squares linear regression and the reciprocal of the squared concentration ($1/x^2$) was used as a weighting factor.
Deviations from the nominal concentrations should be within ± 20% for the LLQ and within ± 15% for the other concentrations.

Accuracy and precision

Accuracy and precision of the assay were determined by analyzing five replicates of QC samples of UDAI at the LLQ, low, mid and high concentration levels together with duplicate calibration standards in three analytical runs. Inter-assay accuracy was calculated as the relative difference between the mean measured concentration after the three runs and the nominal concentration. The accuracy should be within 80-120% for the LLQ and 85-115% for the other concentrations. Intra- and inter-assay precisions were represented by the coefficient of variation (CV%), which should be less than 20% for the LLQ and less than 15% for the other concentrations.

Recovery and ion suppression

For the determination of ion suppression (matrix effect), 1 mL of blank human plasma was processed as a double blank plasma sample. After LLE and evaporation (as described in 'Sample preparation'), the extracts were reconstituted with 50 µL of reconstitution solvent. Three different concentrations of reconstitution solvents (all prepared in acetonitrile-water (50:50, v/v)) were added in triplicate: solvent 1 (3 ng/mL UDAI + 200 ng/mL internal standard), solvent 2 (100 ng/mL UDAI + 200 ng/mL internal standard) and solvent 3 (800 ng/mL UDAI + 200 ng/mL internal standard).
For calculation of ion suppression, the analytical response of the reconstituted extracts was compared with the response of the directly injected reconstitution solvents (1, 2 and 3).
For the extraction recovery study, QC samples (0.15, 5 and 40 ng/mL UDAI) were prepared as described in 'Sample preparation'. The final concentration of UDAI in these QC samples was 3, 100 and 800 ng/mL (due to the 20 times concentration factor during sample preparation).
The analytical response of these QC samples was compared to the response of the blank plasma extracts reconstituted with the solvents 1, 2 and 3 mentioned above.
Determination of the overall recovery was performed by comparing the analytical response of the processed QC samples (0.15, 5 and 40 ng/mL) to the response of unprocessed reconstitution solvents 1, 2 and 3.

Carry-over

Carry-over was assessed by injecting two processed blank plasma samples immediately

after injecting a sample at the ULQ (50 ng/mL). The response of the first blank sample at the retention time of UDAI should be less than 20% of the response of a processed LLQ sample.

Specificity and selectivity

Potential interference between the analyte and endogenous matrix components was investigated by analyzing six batches blank human plasma from different origin. From each batch, a double blank and a LLQ sample were prepared, processed and analyzed according to the described procedures. To assess potential interference between internal standard and analyte, blank samples spiked separately with analyte (at the ULQ) and internal standard were processed and analyzed.

Peak areas of compounds co-eluting with UDAI should be less than 20% of the peak area of the LLQ sample. Areas of peaks that co-elute with the internal standard, should not exceed 5% of the mean internal standard peak area.

The calculated concentrations of the LLQ samples should not deviate more than 20% from the nominal concentrations.

Stability

Stability of stock solutions of UDAI and internal standard was determined by analyzing aliquots of stock solutions in triplicate which were kept at room temperature for 24 h. Peak areas of these samples were compared with those of stock solutions that were stored at -30°C and represented freshly prepared stock solutions. Furthermore, stability of UDAI stock solution was assessed after storage for 2 months at -30°C.

To determine short-term temperature stability, QC samples kept at room temperature for 6 h were analyzed in triplicate and compared to the initial concentrations.

Long-term stability of UDAI was determined in triplicate by analyzing QC samples after storage at -30°C.

Freeze (-30°C)/thaw stability of UDAI in human heparin plasma was assessed by comparing QC samples after three freeze/thaw cycles with the concentrations determined at time zero.

Stability of reconstituted extracts was assessed in quadruplicate after storage for 48 h at 2-8°C and compared with the initial concentrations.

QC samples analyzed in the above mentioned stability experiments were prepared at two concentration levels: 0.15 and 40 ng/mL UDAI. Concentrations of UDAI in the biological matrix samples should not deviate more than 15% from the initial concentrations. UDAI and internal standard were considered stable in stock solution if 95-105% and 80-120% of the initial peak area is recovered for UDAI and benzanilide, respectively.

RESULTS AND DISCUSSION

Mass spectrometry

The protonated molecule of UDAI at *m/z* 232 was used to generate a product ion spectrum. For selected reaction monitoring (SRM), the most abundant fragment ion (*m/z* 105) was selected and the fragmentation conditions were optimized. The conditions of *m/z* 198 to 105 were optimized for monitoring the internal standard benzanilide. MS/MS product scans and the proposed fragmentation pathways of UDAI and benzanilide are depicted in Figure 2.

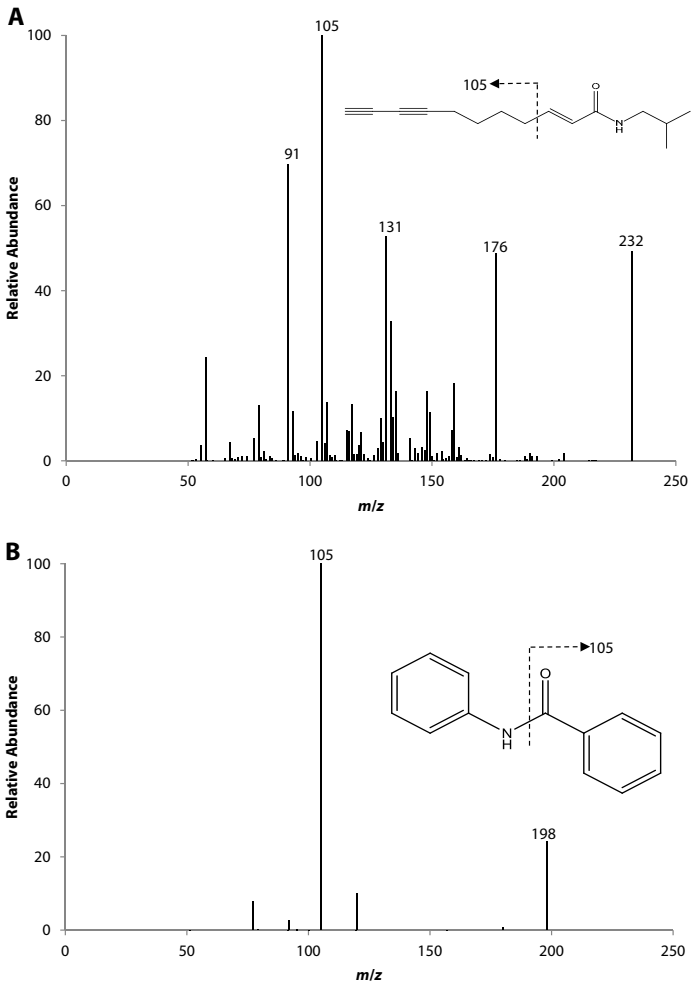

Figure 2. (A) MS/MS product ion scan of undeca-2-ene-8,10-diynoic acid isobutylamide (precursor ion *m/z* 232). (B) MS/MS product ion scan of benzanilide (precursor ion *m/z* 198).

Chromatography

Woelkart *et al.* developed a method using a reversed phase column and the analytes were eluted from the column with a gradient consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) [7-9]. A flow rate of 0.25-0.3 mL/min was used [7-10]. We have tested isocratic elution (A:B (50:50, v/v)) at a flow rate of 0.3 mL/min to shorten the total run time. These conditions resulted in symmetric peaks and a total run time of only 3 min. Representative chromatograms of a blank sample and a human plasma sample spiked at the LLQ are given in Figure 3. In the robustness test, the relative standard deviation of the absolute area of UDAI was less than 2% and less than 5% for the ratio with the internal standard, demonstrating the robustness of the method.

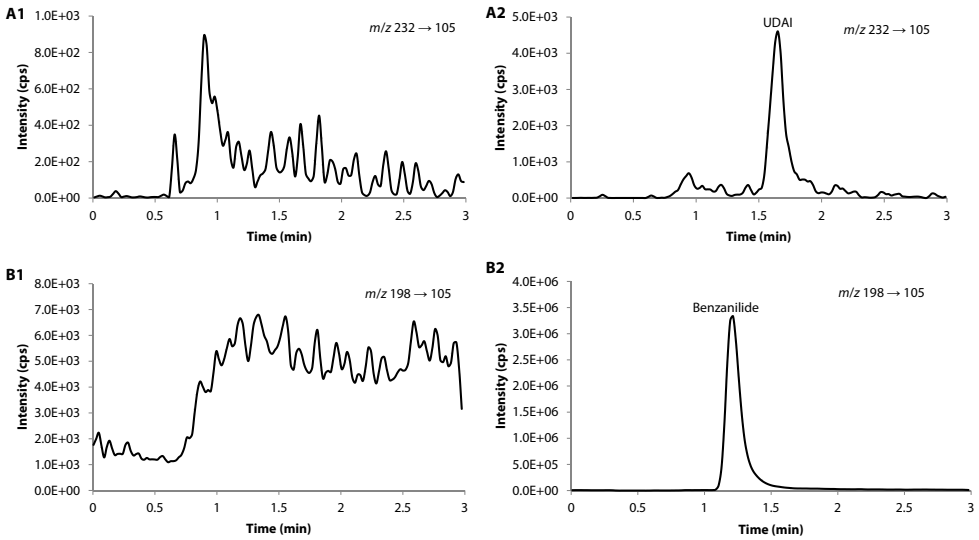

Figure 3. Representative LC-MS/MS chromatograms of a blank human heparinized plasma sample (A1, B1) and of a spiked human heparinized plasma sample at the LLQ level of 0.05 ng/mL (A2, UDAI, t_R = 1.6 min; B2, internal standard benzanilide, t_R = 1.2 min). UDAI = undeca-2-ene-8,10-diynoic acid isobutylamide.

Sample pre-treatment

After administration of a single oral dose of an ethanolic extract of *E. angustifolia* to 11 healthy volunteers, a C_{max} of 1.87 ng/mL in plasma was found for UDAI [7]. This finding indicated the need for a sensitive bioanalytical assay. Clean sample extracts were required to achieve a LLQ of 0.05 ng/mL. LLE was tested in the present study. Several ratios of diethyl ether/n-hexane were investigated: the highest overall recovery was found for diethyl ether/n-hexane (50:50, v/v).

Subsequently, the optimal volume of diethyl ether/n-hexane (50:50, v/v) was assessed by LLE with organic phase volumes ranging from 2 to 9 mL and selecting the volume with the highest absolute MS/MS response of analyte and internal standard. Eight milliliters of

diethyl ether/n-hexane (50:50, v/v) was found to give the best results and was therefore selected.

Validation

Linearity

The assay was linear over a concentration range of 0.05-50 ng/mL for UDAI in human plasma. Using least-squares linear regression (area ratio *versus* the concentration) with a weighting factor of $1/x^2$, the lowest total bias and the most constant bias across the range were obtained. The average regression parameters of the linear regression functions (n = 3) were $y = 7.97 \times 10^{-5} (\pm 6.30 \times 10^{-5}) + 0.0292 (\pm 0.0176)x$ with an average correlation coefficient of 0.994 (± 0.00395). At all concentration levels deviations of measured from nominal concentrations were between -17.9 and 8.15% for LLQ and between -12.2 and 12.1 % at the other concentration levels. CV values varied between 3.46 and 18.1% at LLQ and between 0.148 and 12.4% at the remaining concentration levels.

Accuracy and precision

Assay performance data for UDAI in human plasma are summarized in Table 1. The inter-assay inaccuracy was within ± 12.7% for all concentrations, thus meeting the required ± 15% (± 20% for LLQ) [11]. The within-day and between-day precisions did not exceed 8.23% for all concentrations and therefore fulfilled the required criterion of ± 15% (± 20% for LLQ) [11].

Table 1. Assay performance data for undeca-2-ene-8,10-diynoic acid isobutylamide (n = 15)

Nominal concentration (ng/mL)	Mean measured concentration (ng/mL, ± SD)	Inter-assay accuracy (%)	Within-day precision (% CV)	Between-day precision (% CV)
0.0500	0.0533 (± 0.00454)	107	7.99	8.23
0.150	0.163 (± 0.00686)	108	3.71	4.08
5.00	5.17 (± 0.155)	103	2.56	2.90
40.0	34.9 (± 1.27)	87.3	2.89	3.51

CV = coefficient of variation.

Recovery and ion suppression

Ion suppression has been demonstrated for both UDAI and benzanilide. The mean ion suppression for UDAI was -15.0% (range -22.0 to -8.60%), and for benzanilide a mean ion suppression of -14.1% (range -19.8 to -11.2%) was observed.

After comparing the analytical response of processed QC samples to that of blank processed plasma samples reconstituted with solutions containing analyte and internal standard, mean LLE recoveries of 53.5 and 62.8% were found for UDAI and benzanilide, respectively.

The mean total recovery was 45.5% for UDAI and 53.9% for benzanilide and were found

to be reproducible.

Carry-over

In the first processed blank, the carry-over was 6.84 and 0.687% of the areas of the compounds in a processed LLQ sample for UDAI and benzanilide, respectively. These percentages were far below the required 20% and were thus found to be acceptable.

Specificity and selectivity

SRM chromatograms of six batches of control drug-free heparinized plasma contained no co-eluting peaks > 20% of the analyte peak area at the LLQ level, and no co-eluting peaks > 5% of the area of the internal standard. The six batches showed deviations from the nominal concentrations at the LLQ level between -12.9 and 19.6% for UDAI and were approved.

Further, no cross-analyte/internal standard interference was observed, as no peaks were detected at the retention time of UDAI when analyzing a sample containing only internal standard. Additionally, no peaks were detected at the retention time of benzanilide when a sample containing only UDAI was processed.

Stability

The stability data for UDAI and the internal standard benzanilide are summarized in Table 2. These data show that stock solutions of UDAI and benzanilide were stable for at least 24 hours at ambient temperatures. The stock solution of benzanilide was stable for at least 4 months at -30°C. After storage for 2 months at -30°C, the stock solution of UDAI was just outside the range of 95-105% recovery of the initial measured concentration, indicating that UDAI in stock solution is stable for up to 2 months under the tested conditions.

Short-term stability experiments demonstrated stability of UDAI in human heparinized plasma at ambient temperatures for at least 6 h.

Regarding long-term stability, UDAI was stable in plasma for up to 1 month at -30°C.

Freeze/thaw stability experiments showed that UDAI was stable in human heparinized plasma for at least three freeze (-30°C)/thaw cycles.

Finally, in the final extract stability of UDAI was demonstrated for at least 48 h at 2-8°C.

Table 2. Stability data for undeca-2-ene-8,10-diynoic acid isobutylamide and the internal standard benzanilide

Conditions	Matrix	Initial conc. (ng/mL)	Found conc. (ng/mL)	Dev(%)	CV (%)	No. of replicates
Undeca-2-ene-8,10-diynoic acid isobutylamide						
Ambient, 24 h	Methanol	1.10×10^5	1.14×10^5	4.12	17.4	3
-30°C, 2 months	Methanol	1.10×10^5	1.07×10^5	5.52	3.04	3
Ambient, 6 h	Plasma	0.154 41.2	0.163 44.5	5.58 8.01	6.97 3.03	3 3
-30°C, 1 month	Plasma	0.176 40.4	0.188 44.1	7.28 8.93	13.7 6.25	3 3
3 freeze (-30°C)/thaw cycles	Plasma	0.154 41.2	0.159 40.6	3.31 -1.33	2.53 4.91	3 3
2-8°C, 48 h	Final extract	0.154 5.15 41.2	0.145 4.87 37.5	-6.09 -5.40 -9.07	5.20 1.87 5.66	4 4 4
Benzanilide						
Ambient, 24 h	Methanol	9.70×10^5	9.04×10^5	-6.84	12.1	3
-30°C, 4 months	Methanol	1.07×10^6	1.16×10^6	8.49	3.25	3

CV = coefficient of variation; Dev = deviation from the initial concentration.

APPLICATION OF THE METHOD

To demonstrate the applicability of the present assay, a single pharmacokinetic experiment was conducted in which a human volunteer ingested a single low dose of 20 drops of a commercial extract of *E. purpurea* (Echinaforce®, A. Vogel). The presence of UDAI was detected in the blood sample taken at 60 min (Figure 4A). The identity of UDAI was confirmed by the retention time of the peak at 1.6 min, absent in the blank sample, and which correlates with that of the reference standard of UDAI. Additionally, in the product ion spectrum of *m/z* 232 at 16.0 V the major product ions *m/z* 91, 105, 131 and 176 were observed (Figure 4B) which are identified as those in the reference compound of UDAI (Figure 2A). The plasma concentration of UDAI at t = 60 min was quantified as 0.060 ng/mL. The chromatogram and product ion scan of UDAI in the human sample at t = 60 min are depicted in Figure 4.

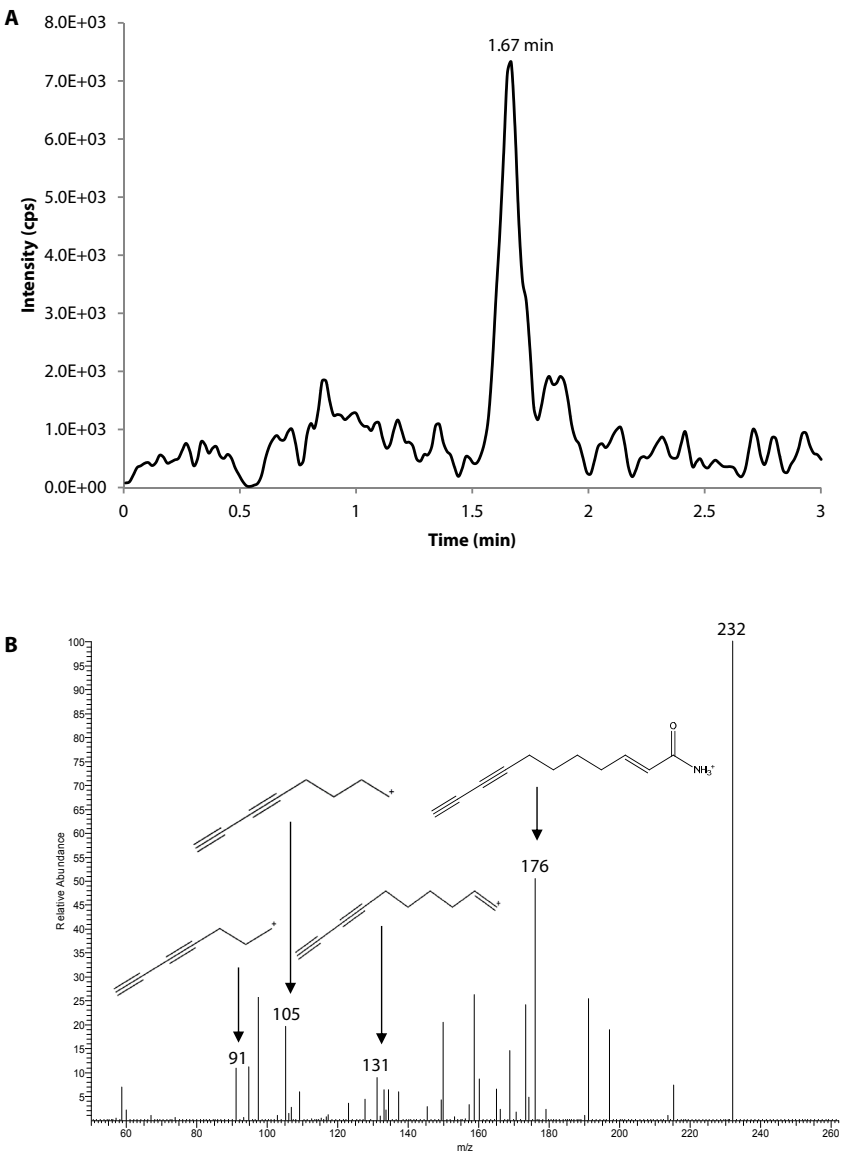

Figure 4. (A) LC-MS/MS chromatogram of a plasma sample taken from a human volunteer 60 min after ingestion of 20 drops of A. Vogel Echinaforce® (t = 60 min). (B) MS/MS product ion scan of undeca-2-ene-8,10-diynoic acid isobutylamide (t_R = 1.67 min) of the human plasma sample at t = 60 min. Proposed chemical structures are depicted for product ions *m/z* 91, 105, 131 and 176.

CONCLUSIONS

This paper describes the development, validation and application of a LC-MS/MS assay for the quantitative analysis of UDAI in human plasma. This is the first quantitative assay for an alkylamide which has been fully validated according to the FDA guidelines on Bioanalytical Method Validation [11]. To extract UDAI from human plasma, a LLE method was performed. Validation results show that this assay is accurate, precise, selective and reproducible. Using 1 mL of plasma aliquots, a concentration range from 0.05 to 50 ng/mL UDAI could be quantified. This sample volume is less than used in previously published LC-MS/MS assays for alkylamides of *E. purpurea* in biological matrices. Furthermore, with a LLQ of 0.05 ng/mL this assay is more sensitive than the previously described bioanalytical assay for UDAI in human plasma [7]. At last, in the pharmacokinetic experiment a peak plasma concentration of 0.06 ng/mL UDAI was measured 60 min after ingestion of 20 drops of a commercial extract of *E. purpurea*. This low concentration of UDAI demonstrated the need for the present, sensitive, assay for UDAI to be used in the clinical study. In our clinical trial, however, we expect to detect higher levels of UDAI in patients, as the patients will use the extract more frequently than our volunteer: 3 times daily 20 drops during a period of 14 days. Thus, this pharmacokinetic experiment showed that the LLQ was sufficient for our purpose to monitor the adherence of *E. purpurea* in a clinical setting.

ACKNOWLEDGEMENTS

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3

CHAPTER 3.2

The bioanalysis of the major *Echinacea purpurea* constituents dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides in human plasma using LC-MS/MS

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ABSTRACT

Background: Alkylamides are a group of active components of the widely used herb *Echinacea purpurea* (*E. purpurea*), which have immunostimulatory and anti-inflammatory effects. For the most abundant alkylamides, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (DTAI), an LC-MS/MS assay has been developed and validated for quantification in human plasma. This assay will be used to support a clinical interaction study with *E. purpurea*.

Materials and Methods: A 300 µL plasma aliquot underwent liquid-liquid extraction with diethylether-n-hexane (50:50, v/v). After evaporization and reconstitution in 100 µL of acetonitrile-water (50:50, v/v) 20 µL of sample were injected into the HPLC system. Chromatographic separation was achieved with a Polaris 3 C18-A column (50 mm x 2 mm ID, particle size 3 µm), a flow rate of 0.3 mL/min and isocratic elution with acetonitrile-water (50:50, v/v) containing 0.1% formic acid during the first 5 min. Hereafter, gradient elution was applied for 0.5 min, followed by restoration of the initial isocratic conditions. The total run time was 7.5 min.

Results and Conclusions: The assay was validated over a concentration range from 0.01 to 50 ng/mL for DTAI, with a lower limit of quantification of 0.01 ng/mL. Validation results show that DTAI can be accurately and precisely quantified in human plasma. DTAI also demonstrated to be chemically stable under relevant conditions. Finally, the applicability of this assay has been successfully demonstrated by measuring the plasma concentration of DTAI in patients after ingestion of a commercial extract of *E. purpurea*.

INTRODUCTION

Nowadays, *Echinacea purpurea* (*E. purpurea*) is one of the most often used herbal medicines. Alkylamides, caffeic acid derivatives, polysaccharides and glycoproteins are considered to be the components responsible for *E. purpurea*'s immunostimulatory and anti-inflammatory effects [1]. For alkylamides, the main lipophilic constituents, seventeen compounds have been identified in *E. purpurea* [2]. The main alkylamides are the isomeric dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (DTAI) [3], which are likely to cross the intestinal barrier [4]. Oral bioavailability of DTAI has been demonstrated in rats [5] and humans [6-9]. Pharmacologically, DTAI have immunomodulatory and anti-inflammatory actions [3, 10, 11], that are presumably mediated by binding to cannabinoid receptors [12].

This article focuses on the quantitative analysis of DTAI in human plasma using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The presented assay will be used to support a clinical study in which the potential pharmacokinetic interaction between *E. purpurea* extract and the anticancer drug docetaxel will be studied. In this clinical study plasma levels will be monitored to assess the adherence of *E. purpurea* intake by the patients and the pharmacokinetics of DTAI.

Previously, few bioanalytical assays for DTAI in human plasma or serum have been published [6-9]. Matthias *et al*. developed an assay for the quantification of eight alkylamides, including DTAI, in human plasma [6]. Drawbacks of this assay, however, are the large amount of solvents used during solid-phase extraction (SPE) and a run time of 23 min. Also, the single ion monitoring (SIM, LC-MS) mode used in this assay is not as specific as *tandem* mass spectrometry (LC-MS/MS).

Sensitive quantitative assays for DTAI in human plasma or serum with a lower limit of quantification (LLQ) down to 0.008 ng/mL have been developed by Woelkart *et al*. [7-9]. However, large sample volumes of up to 16 mL plasma [9] and long run times of 20 min were required for these assays [7-9]. Regarding practical issues such as patient convenience and time efficiency, a faster assay requiring less sample volume was desirable for application in our clinical study. Therefore, our objectives were to develop a sensitive validated assay according to the FDA guidelines on Bioanalytical Method Validation [13], which would require less sample volume and a shorter run time.

MATERIALS AND METHODS

Reagents and chemicals

DTAI ($C_{16}H_{25}NO$, mixture of 2*E*,4*E*,8*Z*,10*Z* and 2*E*,4*E*,8*Z*,10*E* isomers) was purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). The internal standard benzanilide ($C_{13}H_{11}NO$) originated from Acros Organics (Leicestershire, UK). Docetaxel

($C_{43}H_{53}NO_{14}$) and dexamethasone ($C_{22}H_{29}FO_5$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade water, methanol and n-hexane of HPLC quality, acetonitrile of HPLC-S gradient grade quality and analytical grade diethyl ether stabilized with 2,6-di-tert-butyl-4-methylphenol (BHT) were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid was purchased from Merck (Darmstadt, Germany). Blank, drug-free human plasma, containing lithium-heparin as anti-coagulant, was obtained from Sera Laboratories International Ltd. (Haywards Heath, UK). *E. purpurea* drops originated from A. Vogel (Echinaforce®, batch 08K0302, Biohorma BV, Elburg, The Netherlands) and were labeled to contain 95% aerial parts and 5% roots of *E. purpurea* (contents of DTAI and other alkylamides not specified).

Liquid chromatography

The LC-MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-µ pumps (all from Shimadzu, Kyoto, Japan). Sample injections (20 µL) were made on a Polaris 3 C18-A column (50 mm x 2 mm ID, particle size 3 µm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 mm x 2 mm ID, particle size 3 µm, Varian). The column temperature was maintained at 40°C and the autosampler was set at 15°C. Mobile phase A consisted of water, containing 0.2% formic acid, and mobile phase B consisted of acetonitrile. The flow rate was 0.3 mL/min and the total run time was 7.5 min. During the first 5 min of the run, isocratic elution was applied with 50% B. From 5.01 until 5.5 min, the eluent composition was changed to 90% B. Hereafter, the isocratic system of 50% B was restored until the end of the run at 7.5 min.

Mass spectrometry

The Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with electrospray ionization (ESI) operated in the positive ion mode with both quadrupoles set at 0.7 full width at half maximum (FWHM, unit resolution) and with dwell times of 200 ms. For DTAI, the mass transitions from *m/z* 248 to 167 were optimized and for benzanilide, responses from *m/z* 198 to 105 were monitored. The optimized collision energies were -17 V for DTAI and -20 V for benzanilide. Tube lens voltages were 124 V for DTAI and 101 V for benzanilide. Further, spray voltage was set at 4500 V with an ion tube temperature of 210°C. Nitrogen sheath, ion sweep and auxiliary gasses were set at 49, 2.0 and 14 arbitrary units, respectively. Finally, the up-front collision-induced dissociation (CID) was set off and argon collision gas pressure was set at 1.8 mTorr. For data acquisition and processing, Xcalibur software (version 1.4, Thermo Fisher Scientific) was used.

Preparation of stock and working solutions

Two stock solutions of DTAI (1.0 mg/mL) from two independent weightings were prepared

in methanol. One stock solution was used for the preparation of calibration standards (CS), and the other solution was used to prepare quality control (QC) samples. For preparation of CS and QC working solutions, stock solutions were diluted with acetonitrile-water (50:50, v/v). For the internal standard, a stock solution of benzanilide in methanol (1 mg/mL) was diluted with acetonitrile-water (50:50, v/v) to obtain a final working solution of 100 ng/mL benzanilide.
All stock solutions were stored at -80°C until use, while the working solutions were used immediately after preparation.

Preparation of CS and QC samples

CS samples were prepared freshly by spiking CS working solutions to human plasma. Two calibration curves were validated: one curve for low DTAI concentrations (0.01 - 0.05 ng/mL) and another curve for higher DTAI levels (0.05 - 50 ng/mL). The 'low DTAI' curve consisted of CS with concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 ng/mL, while the 'high DTAI' curve included concentrations of 0.05, 0.1, 1, 5, 25 and 50 ng/mL DTAI. For each validation run, these standards were prepared and analyzed in duplicate.
QC samples for the 'low DTAI' curve had concentrations of 0.02 (low), 0.03 (mid) and 0.04 (high) ng/mL DTAI. For the 'high DTAI' curve QC samples were prepared in concentrations of 0.05 (LLQ), 0.15 (low), 5 (mid), 40 (high) and 250 (> upper limit of quantification) ng/mL DTAI. The QC sample with 250 ng/mL DTAI was used for a dilution test above the upper limit of quantification (ULQ). QC samples, prepared in batches, were stored at -30°C until analysis.

Sample preparation

To 300 µL plasma samples, 50 µL of the internal standard benzanilide (100 ng/mL) was added in a 2.0 mL polypropylene reaction tube. After vortex-mixing, liquid-liquid extraction (LLE) was performed with 1.6 mL diethyl ether/n-hexane (50:50, v/v). Subsequently, the samples were shaken with a rotary-mixer for 10 min at 50 rpm. After centrifugation for 10 min at 13,500 rpm (4°C), the samples were stored at -30°C for 60 min. Next, the organic layer was decanted into another 2.0 mL polypropylene reaction tube and evaporated under a stream of nitrogen at 40°C. The dry extract was reconstituted in 100 µL of acetonitrile-water (50:50, v/v). After vortex-mixing, the solution was centrifuged for 10 min at 13,500 rpm (4°C). Finally, the clear supernatant was transferred into a 250 µL glass insert placed in an autosampler vial.

Validation procedures

A full validation of the assay in human heparinized plasma was performed according to the current FDA guidelines on Bioanalytical Method Validation [13]. Parameters that were validated were linearity, accuracy, precision, recovery, matrix effect, specificity, selectivity,

and stability.

Pharmacokinetic application

The applicability of the present assay was assessed in three cancer patients of the clinical interaction study with *E. purpurea* and docetaxel. The clinical study had been approved by the Medical Ethics Committee of the Antoni van Leeuwenhoek Hospital – The Netherlands Cancer Institute and all patients gave written informed consent. The cancer patients ingested 20 drops of a commercial extract of *E. purpurea* (A. Vogel Echinaforce®, batch 08K0302, Biohorma BV, Elburg, The Netherlands) three times daily for fourteen days. After the last ingestion in the morning of day 15, blood samples for pharmacokinetics of DTAI were drawn at t = 0, 30, 60 and 120 min.

RESULTS AND DISCUSSION

Mass spectrometry

The protonated molecule of DTAI at *m/z* 248 was used to generate a product ion spectrum (Supplemental Figure 1A). For selected reaction monitoring (SRM), the most abundant product ion (*m/z* 167) was selected and the collision conditions were optimized. The conditions of *m/z* 198-105 were optimized for monitoring the internal standard benzanilide (Supplemental Figure 1B). MS/MS product ion spectra and the proposed fragmentation patterns of DTAI and benzanilide are depicted in Figures 1A and B.

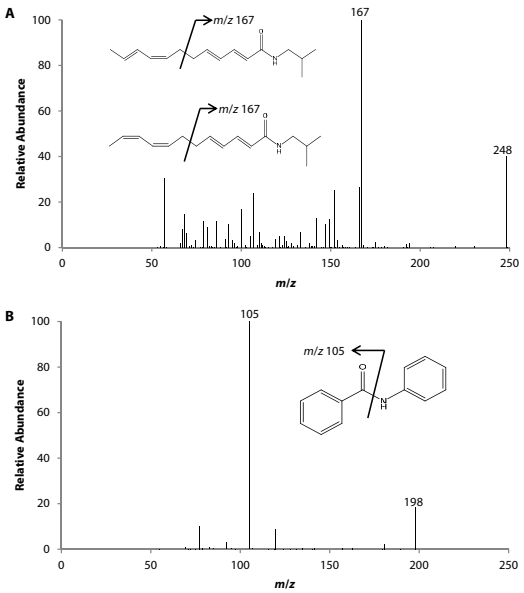

Figure 1. MS/MS product ion spectra of (A) dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides ($[M+H]^+$: *m/z* 248.2 @ -17 V) and (B) benzanilide ($[M+H]^+$: *m/z* 198.1 @ -20 V).

Chromatography

Chromatographic conditions were adopted from our previously validated LC-MS/MS assay for undeca-2-ene-8,10-diynoic acid isobutylamide (UDAI) [14]. At first, an isocratic elution (A:B (50:50, v/v)) at a flow rate of 0.3 mL/min was applied for 5 min. After observing an increasing noise in the MS/MS signal after multiple injections, a gradient was introduced after each injection by rapidly increasing % B from 50 to 90% within 30 s followed by stabilization at 50% B for 2 min. Consequently, the noise was significantly reduced.

In accordance with other bioanalytical assays for DTAI [7-9], the 2*E*,4*E*,8*Z*,10*Z* and 2*E*,4*E*,8*Z*,10*E* isomers were not separated in the present assay. Thus, peak areas of DTAI represent the sum of both isomers.

Representative chromatograms of a double blank sample, a human plasma sample spiked at 0.05 ng/mL DTAI and a patient plasma sample obtained 30 min after ingestion of Echinaforce® extract are given in Figure 2.

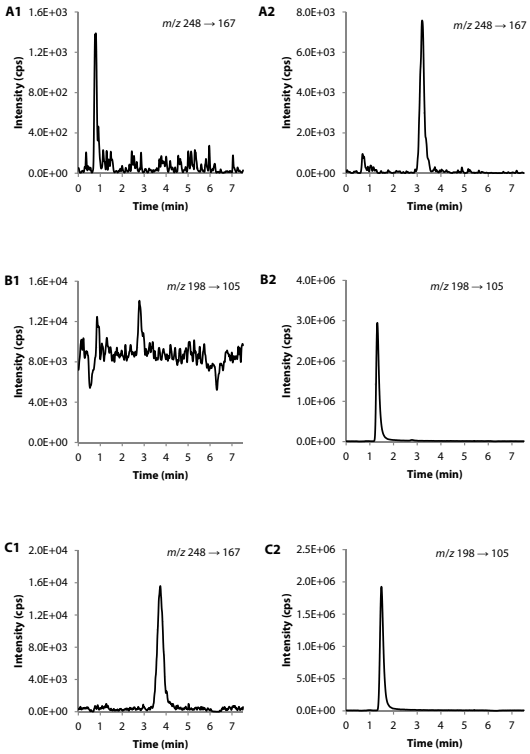

Figure 2. Representative chromatograms of a blank human plasma sample (A1 and B1) and of a human plasma sample spiked at the level of 0.05 ng/mL DTAI (A2, DTAI, t_R = 3.2 min; B2, internal standard benzanilide, t_R = 1.3 min). Representative chromatograms of DTAI (C1) and the internal standard benzanilide (C2) in a patient sample obtained 30 min after intake of 20 oral drops of Echinaforce® extract.
DTAI = dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides.

Sample pre-treatment

LLE was performed with 1.6 mL diethyl ether/n-hexane (50:50, v/v). The composition of the organic phase was optimized in our previous UDAI assay [14], in which the highest overall recovery was found for diethyl ether/n-hexane (50:50, v/v). With a small plasma volume of 300 µL, high and reproducible overall recoveries (87.7%, range 80.4-95.5%) were found for DTAI after LLE with 1.6 mL of organic phase and reconstitution in 100 µL acetonitrile-water (50:50, v/v).

Validation

Linearity

For DTAI concentrations ranging from 0.01 to 0.05 ng/mL and from 0.05 to 50 ng/mL, linearity was assessed by preparation and analysis of duplicate CS samples in three separate runs. The assay was linear over both concentration ranges for DTAI in human plasma. Using least-squares linear regression (area ratio versus the concentration $1/X^2$), the lowest total bias and the most constant bias across the range were obtained. The average regression parameters of the linear regression functions ($n = 3$) for the low and high calibration range were $y = -0.0000905 (\pm 0.0000198) + 0.0171 (\pm 0.0040)x$ ($r^2 = 0.9781 (\pm 0.007937)$) and $y = 0.000375 (\pm 0.000469) + 0.0497 (\pm 0.0174)x$ ($r^2 = 0.9887 (\pm 0.0071784)$), respectively.

At all concentration levels, deviations of measured from nominal concentrations were between -14.9% and 14.7%. Thus, FDA acceptance criteria (± 20% deviation from nominal concentration for LLQ and ± 15% for other concentrations) were met.

Accuracy and precision

Accuracy and precision of the lower level calibration curve (0.01 - 0.05 ng/mL) were validated by analysis of QC samples with DTAI concentrations of 0.02, 0.03 and 0.04 ng/mL (five replicates per concentration level in three analytical runs).

For the higher level calibration curve (0.05 - 50 ng/mL), accuracy and precision of the assay were determined by analyzing five replicates of QC samples of DTAI spiked at 0.05, 0.15, 5 and 40 ng/mL DTAI in three analytical runs.

Assay performance data for DTAI in human plasma are summarized in Table 1. With a within-day inaccuracy ≤ ± 14.0% and a between-day inaccuracy within ± 8.40% for all concentrations, the required inaccuracy criterion of ± 15% (± 20% for LLQ) was met [13]. Within-day and between-day precisions, represented by the coefficient of variation (CV%), did not exceed the required criterion of ± 15% (± 20% for LLQ) [13].

Further, a QC sample > ULQ (250 ng/mL) can be diluted 10 and 100 times in human plasma with acceptable inaccuracy (≤ 15%) and precision (≤ 15%).

Table 1. Assay performance data for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (*n* = 15 per concentration level)

Run	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL, ± SD)	Inaccuracy (%)	Precision (%)	No. of replicates
1	0.0200	0.0197	-1.66	15.0	5
2	0.0200	0.0180	-10.1	14.6	5
3	0.0200	0.0199	-0.407	13.6	5
Between-day	0.0200	0.0192	-4.05	13.6	15
1	0.0300	0.0301	0.237	9.95	5
2	0.0300	0.0323	7.64	14.0	5
3	0.0300	0.0289	-3.69	8.59	5
Between-day	0.0300	0.0304	1.40	11.1	15
1	0.0400	0.0453	13.4	6.41	5
2	0.0400	0.0410	2.50	7.33	5
3	0.0400	0.0411	2.67	12.1	5
Between-day	0.0400	0.0425	6.17	9.23	15
1	0.0500	0.0570	14.0	3.28	5
2	0.0500	0.0566	13.2	6.20	5
3	0.0500	0.0490	-2.00	5.20	5
Between-day	0.0500	0.0542	8.40	8.15	15
1	0.150	0.150	0.133	9.30	5
2	0.150	0.141	-5.87	1.69	5
3	0.150	0.143	-4.93	3.89	5
Between-day	0.150	0.145	-3.56	6.08	15
1	5.00	4.48	-10.4	6.66	5
2	5.00	5.30	5.92	4.51	5
3	5.00	5.00	-0.116	2.09	5
Between-day	5.00	4.92	-1.55	8.02	15
1	40.0	35.7	-10.7	6.61	5
2	40.0	40.6	1.54	11.7	5
3	40.0	37.5	-6.34	2.80	5
Between-day	40.0	37.9	-5.18	9.09	15

Recovery and matrix effect

Based on the mean peak areas of DTAI and benzanilide, the mean matrix effect was 101% (range 96.8 - 106.9%) for DTAI and 92.7% (range 85.5 - 99.2%) for benzanilide. Mean LLE recovery was 86.7% (range 83.1 - 89.3%) for DTAI and 84.0% (range 81.0 - 88.5%) for benzanilide, and mean total recovery was 87.7% (range 80.4 - 95.5%) and 77.7% (range 75.7 - 81.8%) for DTAI and benzanilide, respectively. These results were found to be reproducible and acceptable.

Specificity and selectivity

Potential interference between the analyte and endogenous matrix components was investigated by analyzing a double blank and a sample with 0.05 ng/mL DTAI for six individual batches blank human plasma. The six double blank batches did not contain co-eluting peaks > 20% of the analyte peak area at 0.05 ng/mL DTAI, or co-eluting peaks > 5% of the area of the internal standard. In the six batches spiked at 0.05 ng/mL DTAI, deviations from the nominal concentrations were between -12.5 and -2.36% for DTAI and were approved.

To assess potential interference between the internal standard and analyte, blank samples

spiked separately with analyte (at the ULQ) and internal standard were processed and analyzed. Eventually, no cross-analyte/internal standard interference was observed, as no co-eluting peaks of DTAI and benzanilide were detected.
Assessment of potential interference of co-medication (docetaxel and dexamethasone) revealed no co-eluting peaks > 20% of the DTAI peak area at 0.05 ng/mL or > 5% of the benzanilide peak area in double-blank plasma samples containing only docetaxel (5,000 ng/mL) or dexamethasone (200 ng/mL). Furthermore, the accuracy of plasma samples containing 0.05 ng/mL DTAI were also within accepted ranges in the presence of docetaxel or dexamethasone. Thus, no interference of co-medication was observed.

Stability
The stability data for DTAI are summarized in Table 2. Stability data regarding the internal standard benzanilide have been described previously by our group [14].
Table 2 shows that a stock solution of DTAI was stable for at least 18 h at ambient temperatures and after storage for 3 months at -80°C.
Stability of DTAI in human plasma has been demonstrated at ambient temperatures for at least 18 h, at -30°C for up to 3 months and for at least three freeze (-30°C)/thaw cycles.
Further, stability of DTAI in the final extract and reinjection reproducibility was confirmed after storage for 24 h at 15°C.

Table 2. Stability data for dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides

Conditions	Matrix	Initial conc. (ng/mL)	Found conc. (ng/mL)	Dev(%)	CV (%)
Ambient, 18 h	Methanol	1.00×10^6	9.66×10^5	-3.38	3.69
-80°C, 3 months	Methanol	1.00×10^6	1.02×10^6	2.02	9.29
Ambient, 18 h	Plasma	0.150 40.0	0.150 38.4	-0.113 -4.08	0.408 1.97
-30°C, 3 months	Plasma	0.150 40.0	0.158 39.3	5.41 -1.64	2.80 3.41
3 freeze (-30°C)/thaw cycles	Plasma	0.150 40.0	0.153 41.4	1.89 3.40	8.40 4.25
Processed sample stability (15°C, 24 h)	Final extract	0.150 40.0	0.142 38.4	-5.52 -3.93	6.99 3.17
Reinjection reproducibility (15°C, 24 h)	Final extract	0.150 5.00 40.0	0.149 4.95 38.1	-0.959 -1.11 -4.65	6.61 1.61 3.17

CV = coefficient of variation; Dev = deviation from the initial concentration.

Application of the method

The plasma concentration-time curves of three patients are shown in Figure 3. DTAI could be quantified in all plasma samples and therefore, it is expected that the described assay can be successfully applied in support of the intended clinical interaction study. For all

three patients the plasma concentration-time curves showed a similar time course with a maximum plasma concentration of DTAI achieved at 1 h after ingestion. The measured plasma concentrations were all in the calibration range (0.01 - 50 ng/mL), with 0.181 ng/mL as the maximum and 0.012 ng/mL as the lowest obtained DTAI plasma concentration.

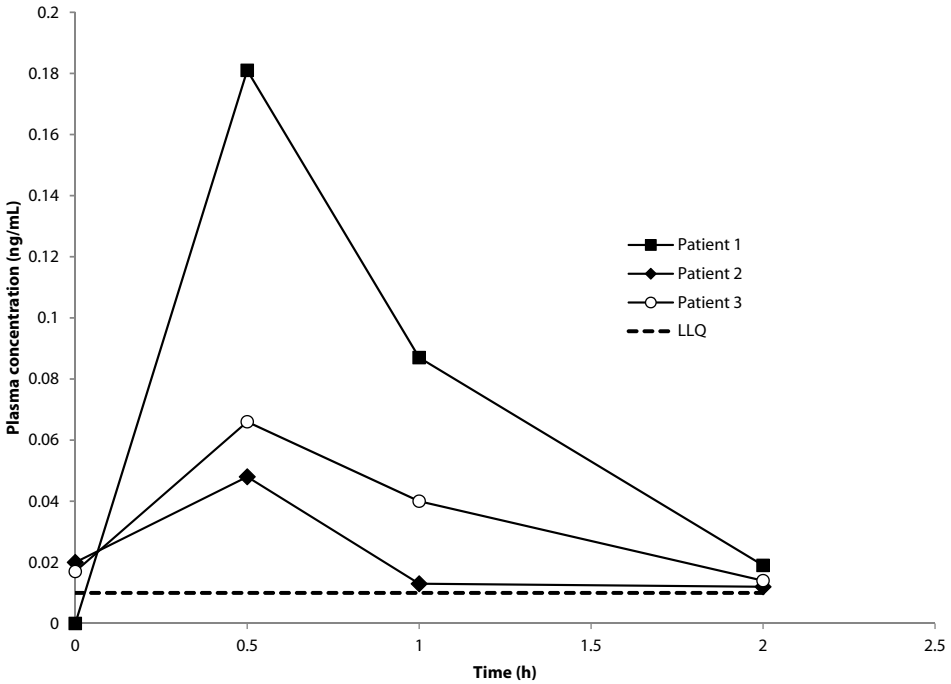

Figure 3. Plasma concentration-time curves of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides in three patients after oral administration of 20 drops of Echinaforce® extract. The dotted line indicates the LLQ of 0.01 ng/mL.

CONCLUSIONS

In this paper, the development, validation and application of a LC-MS/MS assay for the quantification of DTAI in human plasma have been described. Compared to previously published bioanalytical assays for DTAI in human matrix [6-9], this assay requires the lowest sample volume (300 µL plasma) and has the shortest run time (7.5 min). Furthermore, with an LLQ of 0.01 ng/mL this is the most sensitive assay for DTAI that has been validated according to the FDA guidelines on Bioanalytical Method Validation [13]. Finally, the applicability of the present assay has been demonstrated in three patients for the intended clinical study.

ACKNOWLEDGEMENTS

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CHAPTER 3.3

The effect of *Echinacea purpurea* on the pharmacokinetics of docetaxel

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ABSTRACT

Background: The herbal medicine *Echinacea purpurea* has been shown to induce CYP3A4 both *in vitro* and in humans. This study explored whether *Echinacea purpurea* affects the pharmacokinetics of the CYP3A4 substrate docetaxel in cancer patients.

Patients and Methods: Ten evaluable cancer patients received docetaxel (135 mg, 60 min IV infusion) before intake of a commercially available *Echinacea purpurea* extract (20 oral drops 3 times daily) and three weeks later after a 14-day supplementation period with *Echinacea purpurea*. In both cycles pharmacokinetic parameters of docetaxel were determined.

Results: Before and after supplementation of *Echinacea purpurea* the mean area under the plasma concentration-time curve of docetaxel was 3278 ± 1086 and 3480 ± 1285 ng/mL*h, respectively. This result was statistically not significant ($P = 0.51$). Non-significant alterations were also observed for the elimination half-life ($P = 0.56$) and maximum plasma concentration of docetaxel ($P = 0.30$).

Conclusions: *Echinacea purpurea* did not significantly alter docetaxel pharmacokinetics. This result indicates that concomitant supplementation of the applied *Echinacea purpurea* formulation at the recommended dose can be combined safely with docetaxel and presumably also with other anticancer drugs primarily metabolized by CYP3A4.

INTRODUCTION

The use of complementary and alternative medicines (CAM) among cancer patients and the associated risk of herb-drug interactions have increased over the last years [1,2]. Especially for anticancer drugs, which usually have a narrow therapeutic window, these interactions could have serious consequences such as increased toxicity or undertreatment.

Among cancer patients *Echinacea* is a widely used herbal supplement. In a survey including 318 cancer patients *Echinacea* was the most popular herbal medicine, used by 21% of all CAM users [3]. *Echinacea* was also reported to be the second most popular pharmacologic CAM agent among cancer patients enrolled into phase I clinical trials [4].
Echinacea is generally used to stimulate the immune system and to prevent the common cold and upper respiratory infections [5, 6]. The most common species of *Echinacea* are *Echinacea angustifolia*, *Echinacea pallida* and *Echinacea purpurea* (*E. purpurea*). The components of *Echinacea* responsible for the pharmacological effects are caffeic acid derivatives, alkylamides, polysaccharides and glycoproteins [7]. Of these components, caffeic acid derivatives and the more bioavailable alkylamides are found in ethanol liquid extracts for medicinal use [8].

The use of *Echinacea* by cancer patients may interfere with their conventional chemotherapy via interactions with the cytochrome P450 (CYP) 3A4 isoenzyme system. This enzyme system is involved in the metabolism of many anticancer drugs. Both in supersomes and hepatocytes it has been shown that *Echinacea* extracts have the potential to inhibit CYP3A4 *in vitro* [9, 10]. There are indications that *Echinacea* is also capable of inducing CYP3A4. Our group has shown CYP3A4 induction by *E. purpurea* using a gene reporter assay in the human colon adenocarcinoma-derived cell line LS180 (data not shown).
Induction of CYP3A4 by *E. purpurea* has also been shown in healthy volunteers in which the systemic exposure of the CYP3A4 probe midazolam was significantly decreased after supplementation of *E. purpurea* for 28 days [11]. In another clinical study with midazolam in healthy volunteers *E. purpurea* also affected CYP3A4 function[12]. In this volunteer study the systemic clearance of intravenous midazolam was significantly increased which reflects induction of hepatic CYP3A4 activity, while intestinal CYP3A4 was not significantly affected as shown by the lack of significant alterations in oral clearance of orally administered midazolam [12]. In a third clinical study, no significant effect of *E. purpurea* on midazolam pharmacokinetics was reported in healthy volunteers [13]. Thus, both *in vitro* and clinical results showed that *E. purpurea* has the potential to affect CYP3A4, but results concerning inhibition and induction are inconsistent.

An anticancer drug whose systemic exposure may be affected via CYP3A4 modulation by

E. purpurea is docetaxel. Docetaxel has among others been approved for the treatment of locally advanced or metastatic breast, non-small cell lung (NSCLC), and hormone refractory metastatic prostate cancer at doses ranging from 75 to 100 mg/m^2, administrated as a 1-hour infusion every three weeks. Pharmacokinetic interactions between docetaxel and *E. purpurea* could be expected, since docetaxel is extensively metabolized by CYP3A4. As docetaxel is administered intravenously, hepatic CYP3A4 is mainly involved in its metabolism. Expected induction of hepatic CYP3A4 by *E. purpurea* may lead to decreased plasma levels of docetaxel. For docetaxel, systemic exposure has shown to be a good predictor for its efficacy and toxicity [14]. Thus, CYP3A4 induction by *E. purpurea* could lead to undertreatment in patients receiving docetaxel chemotherapy.

Currently, no clinical studies concerning pharmacokinetic interactions between *E. purpurea* and anticancer drugs have been reported. Results of the present study may provide valuable information about the safety of concomitant use of *E. purpurea* with other anticancer agents metabolized by CYP3A4. In the present study, the primary objective was to determine the effect of *E. purpurea* on the pharmacokinetics of docetaxel. In addition, the secondary objective was to assess the effect of *E. purpurea* supplementation on safety parameters such as grade 3 and 4 toxicities induced by docetaxel (according to National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE, version 3.0)).

METHODS

Patients

This clinical study was performed at the Netherlands Cancer Institute (NKI, Amsterdam, the Netherlands). Patients with histological or cytological proof of cancer for whom treatment with docetaxel was considered to be of therapeutic benefit (e.g. advanced breast, gastric, esophagus, bladder, prostate, ovarian, non-small cell lung, head and neck cancer) were included. Other inclusion criteria were: age ≥ 18 years, performance status ≤ 2 according to the World Health Organization (WHO) scale, life expectancy > 3 months, absolute neutrophil count (ANC) ≥ 1.5 × 10^9/L, platelet count ≥ 100 × 10^9/L, hemoglobin level ≥ 6.0 mmol/L, hepatic function as defined by serum bilirubin ≤ 1.5 times the upper limit of normal (ULN) and alanine aminotransferase and aspartate aminotransferase ≤ 2.5 times the ULN, renal function as defined by serum creatinine ≤ 1.5 times ULN or creatinine clearance ≥ 50 mL/min, able and willing to swallow and retain oral medication, to comply to the protocol procedures and to follow dietary restrictions.

Patients were excluded in case of any treatment with investigational drugs within thirty days before the start of the study or the use of herbal supplements within six weeks prior

to study treatment. Other exclusion criteria were: alcoholism, drug addiction, psychotic disorders leading to non-adequate follow-up, concomitant use of multidrug resistance (MDR) and CYP3A modulating drugs, uncontrolled infectious disease, HIV-1 or HIV-2 type patients, unresolved (> grade 1) toxicities of previous chemotherapy, bowel obstruction or motility disorders that may influence the absorption of drugs, pregnancy, chronic use of H2-receptor antagonists or proton pump inhibitors, neurologic disease that may render a patient at increased risk for peripheral or central neurotoxicity and presence of symptomatic cerebral or leptomeningeal metastases.
The study (EudraCT number: 2008-000886-41) was approved by the Medical Ethical Committee of the NKI and all patients provided written informed consent prior to study entry. All patients were treated between April 2009 and March 2010.

Drug administration

Docetaxel (Taxotere®, Aventis Pharma S.A., Antony Cedex, France) was administered intravenously and was supplied in a 15 mL clear glass vial containing 2 mL of a 40 mg/mL docetaxel solution in polysorbate 80. Standard docetaxel pre-treatment consisted of oral dexamethasone 8 mg two times daily for three consecutive days: one day before, on the day of docetaxel administration and one day after. Further, commercially available *E. purpurea* drops were used (A. Vogel Echinaforce®, batch 08K0302, Biohorma BV, Elburg, The Netherlands). These drops were labeled to contain 95% aerial parts and 5% roots of *E. purpurea*.

Study design and procedures

On day 1, all patients received docetaxel at an absolute dose of 135 mg given as a 60 min intravenous administration (cycle 1). The dose of 135 mg was based on a safe dose of 75 mg/m^2 and a mean body surface area of 1.8 m^2. From day 7 until the morning of day 22, the patients ingested 20 drops of the *E. purpurea* extract three times daily. On day 22, the second cycle of docetaxel was administered according to the same dosing schedule as on day 1 (Figure 1). The follow-up of each patient ended with an end of treatment visit three weeks after day 22.

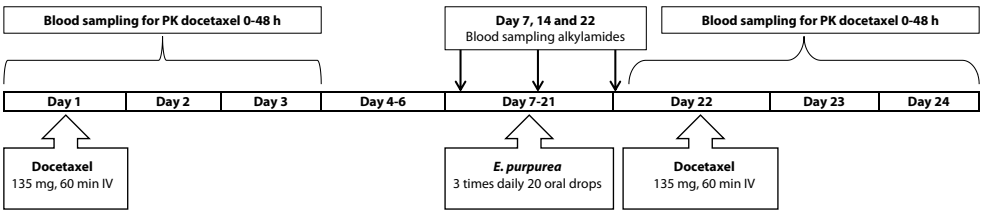

Figure 1. Study design.
IV = intravenous; PK = pharmacokinetics.

Docetaxel analysis

Blood samples for assessment of docetaxel pharmacokinetics were drawn at pre-dose, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 7, 10, 24 and 48 h after start of the docetaxel infusion. Blood was collected in heparinized tubes and centrifuged at 1500 x *g* for 10 min at 4°C. Subsequently, plasma was separated and stored at -20°C until analysis.

Docetaxel plasma levels were quantified using a validated liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) assay with a lower limit of quantification (LLOQ) of 0.25 ng/mL [15].

Analysis of *E. purpurea* constituents: alkylamides

In order to check the compliance to *E. purpurea* intake, patients had to keep diaries, they were called at regular intervals by the research team and single blood samples were collected in heparinized tubes on day 7, day 14 and day 22. In a subset of four patients, the pharmacokinetics of alkylamides were studied by collection of blood samples on t = 0, 0.5, 1 and 2 h after administration of *E. purpurea*. Plasma was separated after centrifugation at 1500 x *g* for 10 min at 4°C and stored at -20°C until quantitative analysis of dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides (DTAI) using a validated LC-MS/MS assay [16].

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using non-compartmental analysis with *R* software (version 2.10.1, *R* Development Core Team, Vienna, Austria) by employing validated scripts.

The following pharmacokinetic parameters of docetaxel were calculated: area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$) and maximum plasma concentration (C_{max}).

Statistical analysis

For each patient, the $AUC_{0-\infty}$, $t_{1/2}$ and C_{max} of docetaxel in cycle 1 (before *E. purpurea*) were compared with values obtained in cycle 2 (after *E. purpurea)*. After logarithmic transformation of these parameters, a paired Student's *t* test ($\alpha = 0.05$) was performed by use of *R*.

Docetaxel-related adverse events

Docetaxel-related adverse events during cycle 1 and 2 were registered according to NCI CTCAE version 3.0. Adverse events were considered as docetaxel-related when rated as possibly, probably or definitely related by the investigator.

RESULTS

Patients

Eleven patients were included (Table 1), of which one patient needed to be replaced as the second docetaxel course was not administered due to her deteriorated physical condition. Hence in total ten patients were eligible for evaluation.

Table 1. Baseline patient characteristics (n = 11)

Gender		
	Female	2
	Male	9
Age (years)		
	Median	58
	Range	42-67
Race		
	Caucasian	11
WHO Performance status		
	0	5
	1	3
	2	3
Primary tumor		
	Ovarian	5
	Non-small cell lung carcinoma (NSCLC)	2
	Endometrium	1
	Unknown primary tumor	2
	Esophageal	1

Effect of *E. purpurea* on the pharmacokinetics of docetaxel

The individual differences in docetaxel $AUC_{0-\infty}$ are depicted in Figure 3. In five patients an increase of the $AUC_{0-\infty}$ of docetaxel was observed after *E. purpurea* supplementation, while in the other five patients the $AUC_{0-\infty}$ decreased. In Figure 2 the mean plasma concentration time curves of docetaxel in the absence and presence of *E. purpurea* are presented. After supplementation of *E. purpurea* the mean docetaxel $AUC_{0-\infty}$ increased from 3278 ± 1086 to 3480 ± 1285 ng/mL*h. This result, however, was statistically not significant (P = 0.51). The mean $t_{1/2}$ before and after supplementation was 30.8 ± 19.7 h and 25.6 ± 5.9 h (P = 0.56), respectively. Further, C_{max} of docetaxel was 2224 ± 609 ng/mL in cycle 1 and 2097 ± 925 ng/mL in cycle 2 (P = 0.30). These pharmacokinetic parameters were also statistically not significant.

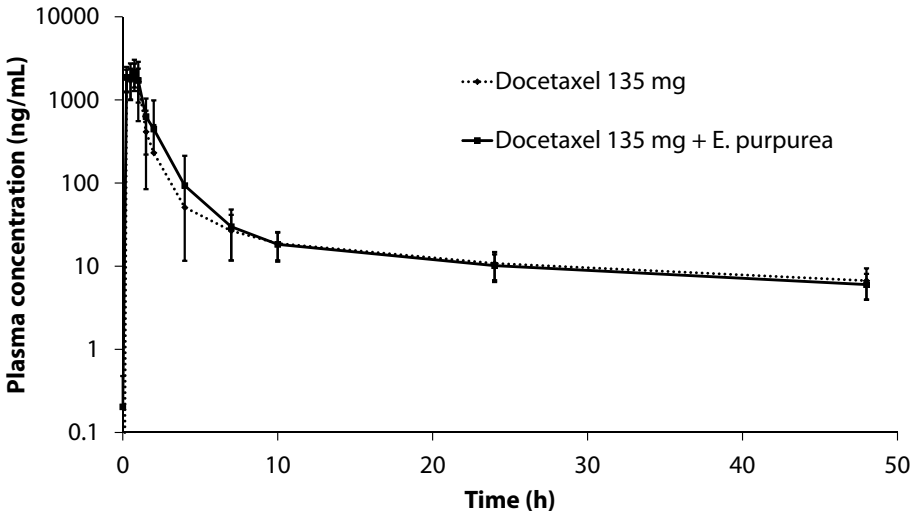

Figure 2. Mean (±SD) plasma concentration-time curves for docetaxel before and after *E. purpurea* supplementation ($n = 10$).

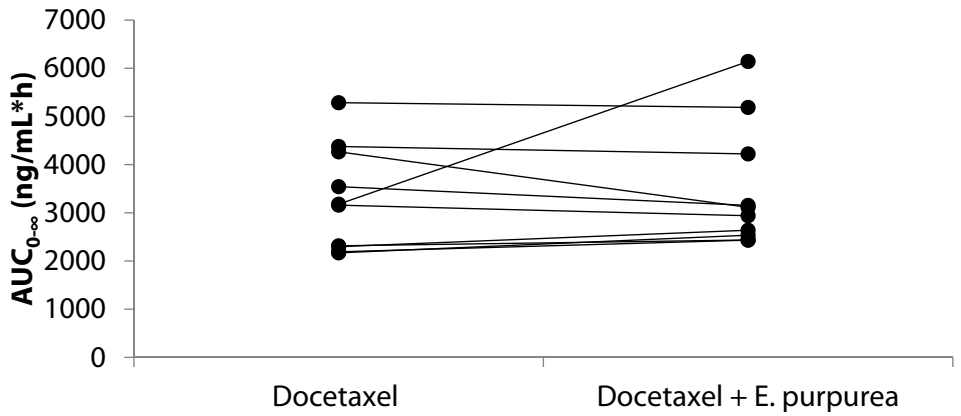

Figure 3. Individual $AUC_{0-\infty}$ values of docetaxel before and after *E. purpurea* supplementation ($n = 10$). $AUC_{0-\infty}$ = area under the docetaxel plasma concentration - time curve extrapolated to infinity.

Docetaxel-related adverse events

The incidence of docetaxel-related adverse events differed between the two docetaxel courses (Table 2). During the course without *E. purpurea* 24 adverse events (grade 1-2: 22 events; grade 3-4: 2 events) were reported in nine patients, while 16 adverse events (grade 1-2: 15 events; grade 3-4: 1 event) occurred in six patients in the course after *E. purpurea* supplementation. However, in the majority of the patients the incidence of docetaxel-related adverse events did not correlate with changes in the $AUC_{0-\infty}$ of docetaxel.

Table 2. Incidence of docetaxel-related adverse events (NCI CTCAE version 3.0)

	Cycle 1 (without *E. purpurea*, *n* = 10)		Cycle 2 (with *E. purpurea*, *n* = 10)	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Patients with adverse events, *n*	9	1	6	1
Adverse events, *n*				
Fatigue	5	-	4	-
Alopecia	5	-	5	-
Rash (erythema, pruritus/itching)	3	-	1	-
Allergic reaction	2	-	1	-
Diarrhea	2	-	-	-
Mucositis/stomatitis	-	-	2	-
Leukopenia	-	1	-	-
Neutropenia	-	1	-	-
Hemoglobin decreased	-	-	1	1
Constipation	1	-	-	-
Taste alteration	1	-	-	-
Myalgia	1	-	-	-
Nausea (heartburn/dyspepsia)	1	-	-	-
Hyperbilirubinemia	1	-	-	-
Sensory neuropathy	-	-	1	-
Total adverse events, *n*	**22**	**2**	**15**	**1**

Adherence to *E. purpurea* intake

Adherence was confirmed by inspection of patients' diaries, telephone calls and inspection of returned bottles. Bioanalysis of alkylamides in plasma samples was less applicable to demonstrate adherence. There are several bioanalytical assays for quantification of alkylamides such as undeca-2-ene-8,10-diynoic acid isobutylamide [17] and DTAI [16]. In the present study DTAI, the most abundant alkylamides in *E. purpurea*, were determined. Unfortunately, in the majority of the patients' samples collected at random time points on day 14 and 22, DTAI plasma levels were below the LLOQ of 0.01 ng/mL. Pharmacokinetic blood sampling from 0 until 2 hours after ingestion of *E. purpurea* revealed that DTAI reached the LLOQ already after 2 hours (data not shown). This finding indicates that the absence of DTAI in plasma samples collected during the study was caused by the low systemic absorption and rapid elimination of DTAI.

DISCUSSION

Based on significant induction of CYP3A4 by *E. purpurea* in previous clinical studies with midazolam [11, 12] and in our gene reporter assay with LS180 cells (data not shown), the pharmacokinetic interaction between *E. purpurea* and docetaxel was investigated in the present clinical study. *E. purpurea* did not significantly affect systemic exposure to

docetaxel in the current study. Intraindividual changes in AUC (Figure 3) were in line with an estimated intraindividual variability in docetaxel clearance of 25% [18]. There are no clear explanations for the remarkable 93% increase in $AUC_{0-\infty}$ in one patient. The incidence of docetaxel-related adverse events, however, did not increase in this patient (data not shown).

Compared to the clinical studies in which significant induction of CYP3A4 by *E. purpurea* was found using midazolam as CYP3A4 probe [11, 12], our study differed in formulation, dose and dosing regimen which may explain the divergent outcome. First, in the study of Penzak *et al.* [11], *E. purpurea* was administered for a longer time period (28 days). In the study of Gorski *et al.* [12], however, a shorter supplementation period (8 days) was applied but the dosing frequency was higher (4 times daily).

Second, comparison between the contents of the *E. purpurea* formulations used in the midazolam studies [11,12] and in the present study is complicated. The formulations of Penzak *et al.* [11] and Gorski *et al.* [12] contained 500 mg and 400 mg *E. purpurea* extract, respectively. Our commercial product was only labeled to contain 95% aerial parts and 5% roots of *E. purpurea* and no information was provided about the total amount of extract used. This formulation has been shown to contain 18 – 34 µg/mL DTAI [5, 9]. In contrast, contents of DTAI or other alkylamides in the extracts used in the midazolam studies [11, 12] were not specified.

Besides differences in the amounts and phytochemical content of *E. purpurea* extracts, also differences in their origin may have contributed to the conflicting clinical outcomes . For example, alkylamide content is known to vary considerably across different parts of *E. purpurea* plants [19] and DTAI are more abundant in roots than in leaves. Consequently, the root extract used by Gorski *et al*. [12] was likely to contain more DTAI than our product and could exert a more potent effect on CYP3A4. The ability of alkylamides and *E. purpurea* extracts to induce CYP3A4, however, is inconclusive according to *in vitro* data. Recently, Modarai *et al*. have shown that *E. purpurea* extract and isolated alkylamides did not significantly induce CYP3A4 in HepG2 cells [20]. However, the lack of an effect on CYP3A4 could be explained by the use of HepG2 cells. It has previously been shown that LS180 cells are to be preferred over HepG2 cells to study CYP3A4 induction, because LS180 cells show higher CYP3A4 expression [21]. In LS180 cells our group has shown a significant induction of CYP3A4 by isolated alkylamides and *E. purpurea* extracts using a gene reporter assay, which is a reliable method to assess the CYP3A4 induction potential of compounds [22]. Induction of CYP3A4 became significant ($P < 0.05$) at relatively high concentrations of 10 and 100 µg/mL alkylamides and *E. purpurea* extract (data not shown).

In addition to the moderate CYP3A4 inducing properties of *E. purpurea*, its systemic exposure could be insufficient to significantly induce hepatic CYP3A4 in the present

study. For example, pharmacokinetic analysis of DTAI indicated that plasma levels of these major alkylamides were undetectable or in the lower range of the calibration curve (< 0.08 ng/mL) halfway the supplementation period. In addition, DTAI were also rapidly eliminated within two hours after intake. As plasma levels of DTAI were not or hardly quantifiable throughout the study period on day 14 and 22, compliance to *E. purpurea* supplementation could not be checked by pharmacokinetic analysis of DTAI. However, inspection of patient diaries and returned bottles of *E. purpurea* indicated that patients ingested their drops according to schedule.

Besides the dosing regimen and content of the applied *E. purpurea* product, docetaxel pre-treatment with dexamethasone may also have contributed to the lack of a significant effect of *E. purpurea* on docetaxel pharmacokinetics. Dexamethasone is a known inducer of CYP3A4 [23]. Assuming induction of CYP3A4 by dexamethasone, systemic exposure to docetaxel could have already been decreased in both courses, thus making the inductive effect of *E. purpurea* during the second course less noticeable. However, results regarding clinical effects of dexamethasone on CYP3A4 are conflicting. A significant pharmacodynamic interaction has been shown between dexamethasone and the CYP3A4 substrate lapatinib [24], while dexamethasone did not significantly alter docetaxel pharmacokinetics in Asian patients [25]. Presumably, these differences in outcomes resulted from differences in exposure to dexamethasone. In the lapatinib study the median duration of treatment with dexamethasone was eleven days [24], which was substantially longer than the three-day treatment period with dexamethasone in the study with docetaxel in Asians [25]. These data suggest that treatment with dexamethasone for three days in the present study would only have had a modest inductive effect on CYP3A4.

It should be noted that the outcome of this study only applies to the specific *E. purpurea* formulation and dose used in the present study. As stated above, alkylamide distribution varies in different parts of *E. purpurea* plants [19] and also in several liquid *E. purpurea* preparations [26]. Thus, the risk of CYP3A4-mediated interactions may be product-dependent.

In conclusion, our findings showed that at the recommended dose and schedule of a commercially available *E. purpurea* extract no statistically significant interference with docetaxel pharmacokinetics could be demonstrated. Therefore, concomitant use of the investigated herb-drug combination can be considered safe.

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3

CHAPTER 3.4

Inter-batch analysis of alkylamides in a commercial *Echinacea purpurea* liquid extract

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ABSTRACT

Background: In commercially available ethanolic extracts of the herbal immunostimulant *Echinacea purpurea*, alkylamides are the main lipophilic and bioavailable constituents. Alkylamides have been shown to interact with cytochrome P450 (CYP) enzymes and their composition can vary considerably between different commercial *Echinacea purpurea* extracts. Less attention, however, has been addressed to alkylamide variability between commercial extracts of one brand. Therefore, this study has compared the alkylamide content in five batches of a popular commercial *Echinacea purpurea* extract in the Netherlands. One of these batches was used in a clinical interaction study, thus inter-batch comparison of alkylamide content would indicate whether the clinical results are applicable to other batches of the commercial extract.

Materials and Methods: For this quality control analysis a method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used. Using alkylamide reference standards and data published in literature, alkylamides were identified based on retention time and mass spectrometric analysis.

Results and Conclusions: Results show that all alkylamides which were reported previously in *Echinacea purpurea* were present in the five tested batches. Further, the alkylamide content in these batches were comparable, suggesting that the result of the clinical interaction study can be extrapolated to other batches of the *Echinacea purpurea* extract.

INTRODUCTION

Echinacea purpurea (*E. purpurea*) is one of the most popular used herbal medicines worldwide. Caffeic acid derivatives, polysaccharides and alkylamides are associated with the immunostimulant properties of *E. purpurea*. In commercially available ethanol/water extracts of *E. purpurea* alkylamides are the major bioavailable constituents [1]. Nowadays, 18 different alkylamides have been identified in *E. purpurea* [1, 2].

Besides exerting pharmacological activity, alkylamides also influence cytochrome P450 (CYP) enzymes *in vitro* and may thus cause pharmacokinetic interactions with other CYP substrate drugs in clinical practice. Therefore, our group has investigated the pharmacokinetic interaction between a commercial *E. purpurea* extract (Echinaforce®, A. Vogel) and the anticancer drug docetaxel in patients with advanced cancer (article submitted for publication). The main outcome of this study was that the tested *E. purpurea* extract did not significantly alter docetaxel pharmacokinetics. However, relatively little information was publicly available about the phytochemical content of the applied *E. purpurea* formulation. The product label only revealed that the extract contained 95% aerial parts and 5% roots of *E. purpurea*. No information was publicly available concerning the amounts of alkylamides, caffeic acid derivatives, polysaccharides and glycoproteins.

Previously, it has been shown that alkylamide content can vary considerably between several commercial *E. purpurea* products [1, 3-5]. This variation can be introduced by several factors, such as the used plant parts [6], formulation of the *E. purpurea* product [4], geographic origin [1], and growing conditions (wild *vs* cultivated) [7]. Since alkylamide content has been shown to correlate with the potency of *E. purpurea* extracts to inhibit CYP enzymes [5], the relevance of CYP-mediated interactions and the pharmacological potency may depend on the extract used. Variation in alkylamide content also complicates the extrapolation of conclusions of interaction studies to other extracts of the same herbal product. Therefore, it is possible that supplementation of other brands of *E. purpurea* liquid extracts or other Echinaforce® batches would have different effects on docetaxel pharmacokinetics than in our clinical study.

To address this issue, the present study concerned a quality control analysis of five batches of Echinaforce®. Previous quality control studies of alkylamides in commercial *E. purpurea* formulations have predominantly focused on products from different manufacturers [1, 3-5]. In this analysis, we have focused on an inter-batch comparison of alkylamide content in Echinaforce® liquid extracts from the same supplier, including the batch used in our clinical trial. For the identification of alkylamides the most comprehensive list of reported alkylamides in *E. purpurea* has been used (Table 1) [1, 2].

Table 1. Alkylamides reported in *E. purpurea* [1, 2]

No.	Alkylamide	*m/z* [M + H]$^+$
1	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	230
2	Undeca-2*E*-ene-8,10-diynoic acid isobutylamide	232
3	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	230
4	Dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	244
5	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	244
6	Dodeca-2*E*-ene-8,10-diynoic acid isobutylamide	246
7	Dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide	246
8	Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	244
9	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide	244
10	Trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	258
11	Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	258
12	Dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide	260
13	Dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide	248
14	Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide	248
15	Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	262
16	Dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	250
17	Pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide	286
18	Dodeca-2*E*,4*E*-dienoic acid isobutylamide	252

MATERIALS AND METHODS

Reagents and chemicals

Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides ($C_{16}H_{25}NO$, mixture of 2*E*,4*E*,8*Z*,10*Z* and 2*E*,4*E*,8*Z*,10*E* isomers) were purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). Undeca-2*E*-ene-8,10-diynoic acid isobutylamide, dodeca-2*E*-ene-8,10-diynoic acid isobutylamide and dodeca-2*E*,4*E*-dienoic acid isobutylamide originated from ChromaDex, Inc (Irvine, CA, USA). The internal standard benzanilide was purchased from Acros Organics (Leicestershire, UK). LC-MS grade water, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid was purchased from Merck (Darmstadt, Germany). *E. purpurea* drops originated from A. Vogel (Table 2, Echinaforce®, batches 08K0302, 10A0603, 12C2903, 12H2002 and 12H2702, Biohorma BV, Elburg, The Netherlands) and were labeled to contain 95% aerial parts (herba) and 5% roots (radix) of *E. purpurea*.

Table 2. Analyzed Echinaforce® batches

Batch Echinaforce®	Batch number	Expiration date
1	08K0302	October 2013
2	10A0603	October 2014
3	12C2903	January 2017
4	12H2002	June 2017
5	12H2702	June 2017

Equipment

Liquid chromatography was carried out using an Accela quaternary pump and autosampler. Sample injections (20 µL) were made on a Polaris 3 C18-A column (50 x 2 mm ID, particle size 3 µm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 x 2 mm ID, particle size 3 µm, Varian). The column temperature was maintained at 40°C and the autosampler was set at 4°C.

Liquid chromatography conditions were adopted from the analysis of Mudge *et al*.[1]. In brief, mobile phase A consisted of water containing 0.1% formic acid, and mobile phase B was composed of acetonitrile. At a flow rate of 0.5 mL/min the following gradient elution was applied: 0 - 4 min, 39 - 40% B; 4 - 8 min, 40 - 50% B; 88 - 10.5 min, 50 - 70% B, 10.5 - 11.5 min, 70% B; 11.5 - 12 min, 70 - 39% B; 12 - 15 min, 39% B.

Mass spectrometry analysis was performed on a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with heated electrospray ionization (HESI), which operated in the positive ion mode with both quadrupoles set at 0.7 full width at half maximum (FWHM, unit resolution) and with dwell times of 100 ms.

Optimization of the mass spectrometry conditions was performed by direct infusion of the reference standard of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (22.3 µg/mL in methanol/water (70:30, v/v)). Tube lens voltage was optimized at 103 V. Further, spray voltage was set at 4000 V with a vaporizer temperature of 261°C. Nitrogen sheath, ion sweep and auxiliary gasses were set at 60, 4.0 and 35 arbitrary units, respectively. Capillary temperature was 216°C and the collision gas pressure was set at 1.0 mTorr.

Mass spectrometry scans were carried out using a data dependent scan method, which consisted of a full MS scan (scan range *m*/*z* 195-305) alternating with MS/MS scans of present protonated alkylamide molecules at a collision energy of -15 V. MS/MS analysis was performed on the 18 precursor *m*/*z* $[M + H]^+$ listed in Table 1. Obtained product ion spectra were used to select an appropriate product ion for alkylamide quantification with selected reaction monitoring (SRM). In the analysis of multiple Echinaforce® batches, SRM analysis resulted in a peak area ratio (area alkylamide *vs* area internal standard) for each alkylamide, which was compared to its response in batch 1. For data acquisition and processing, Xcalibur software (version 2.0.7, Thermo Fisher Scientific) was used.

Sample preparation

For the identification of alkylamides, methanolic stock solutions of the alkylamide reference standards were diluted in methanol-water (70:30, v/v) to obtain final concentrations of 100 µg/mL dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (alkylamides **13** and **14**), 10 µg/mL undeca-2*E*-ene-8,10-diynoic acid isobutylamide (alkylamide **2**), 50 µg/mL dodeca-2*E*,4*E*-dienoic acid isobutylamide (alkylamide **18**) and 50 µg/mL dodeca-2*E*-ene-8,10-diynoic acid isobutylamide (alkylamide **6**). Then, 20 µL of these standards were injected

separately and the retention times, $[M + H]^+$ and product ion spectra were recorded and used to identify the presence of these alkylamides in the extracts.
Samples of Echinaforce® extracts were prepared by adding 100 µL of the internal standard benzanilide (100 ng/mL in methanol-water (70:30, v/v)) to 100 µL of each extract. Then, this mixture was centrifuged for 5 min at 13,500 x *g* (4°C). Subsequently, 20 µL of the clear supernatant was injected into the HPLC system.

Identification of alkylamides

Extracted ion chromatograms of each *m/z* $[M + H]^+$ in Table 1 were studied. Subsequently, the product ion spectrum of the *m/z* $[M + H]^+$ were used to evaluate the fragmentation pattern of the alkylamide. Previously reported fragmentation patterns [2] and products [1, 2] of alkylamides were useful for the assignment of the alkylamides.
Identification of alkylamides of which reference standards were available, was facilitated by comparing the retention time and product ion spectrum of the *m/z* $[M + H]^+$ in the reference standard with that of the extract. Isomeric alkylamides were distinguished by previously determined relative elution order [1].

RESULTS AND DISCUSSION

Identification of alkylamides in the study batch of Echinaforce®

Table 3 shows the alkylamides (no. **1** - **18**) that were present in the Echinaforce® batch used in the clinical study. Alkylamides listed in Table 1 were assigned to peaks (A – Q) in the total ion chromatogram (Figure 1).

Peaks A and C

The identity of the isomeric pair undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (alkylamide **1**) and undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (alkylamide **3**) was confirmed by the presence of *m/z* 230 $[M + H]^+$, retention time and the presence of the product ions *m/z* 157 (acyllium ion), *m/z* 129 (alkyl chain by loss of isobutylamide), *m/z* 131 (alkyl chain by loss of isobutylamide and saturation double bond), *m/z* 174 (loss isobutyl group). The fragmentation patterns for these product ions are depicted in Figure 2. A more detailed description of these patterns are reported by Spelman *et al.* [2]. Distinction between the isomers was based on previously reported elution order in which the *E*,*Z* isomer (peak A) eluted prior to the *Z*,*E* isomer (peak C) [1, 8].

Table 3. Assignment of alkylamides in Echinaforce® (batch1)

Peak	No. alkylamide	Alkylamide	SRM transition	t_R (min)	*m/z* ions in product ion spectrum
A	1	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	230 → 129	2.2	174, 157, 131, 129, 91
B	2	Undeca-2*E*-ene-8,10-diynoic acid isobutylamide*	232 → 91	2.6	176, 159, 133, 131, 105, 91
C	3	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	230 → 129	2.7	174, 157, 131, 129, 91
D	4	Dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	244 → 145	3.0	188, 171, 145, 143, 128, 117
E	5	Undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	244 → 131	3.3	174, 157, 131, 129, 91
F	6 7?	Dodeca-2*E*-ene-8,10-diynoic acid isobutylamide* Dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide?	246 → 105	3.8	190, 167, 147, 145, 105
G	8	Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	244 → 145	3.9	188, 171, 145, 143 , 128, 117
H	9	Undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide	244 → 131	4.3	202, 174, 157, 131, 129
I	10	Trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	258 → 157	4.6	202, 185, 157, 131, 129, 117
J	11	Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	258 → 188	4.8	188, 145, 143, 128, 117
K	12	Dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide	260 → 173	5.6	190, 173, 147, 145, 105
L	13	Dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide*	248 → 167	6.0	175, 167, 152, 149, 147, 142, 133, 107, 57
M	14	Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide*	248 → 167	6.0	175, 167, 152, 149, 147, 142, 133, 107, 57
N	15	Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	262 → 181	7.7	181, 175, 149, 147
O	16	Dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	250 → 167	7.8	194, 177, 167, 152, 149
P	17	Pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide	286 → 145	8.2	230, 213, 185, 145, 143, 129, 117, 105
Q	18	Dodeca-2*E*,4*E*-dienoic acid isobutylamide*	252 → 196	9.6	196, 179, 95

* reference standard utilized for identification

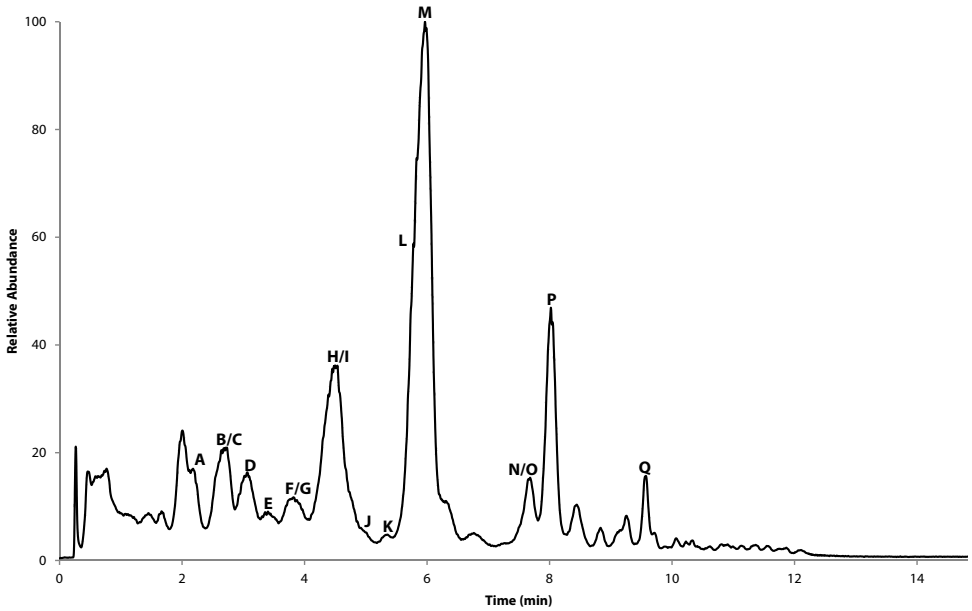

Figure 1. Total ion chromatogram of Echinaforce® batch 1 obtained with LC-MS analysis (mass range *m/z* 229 - 301. Peak assignments correspond to the designations in Table 3.

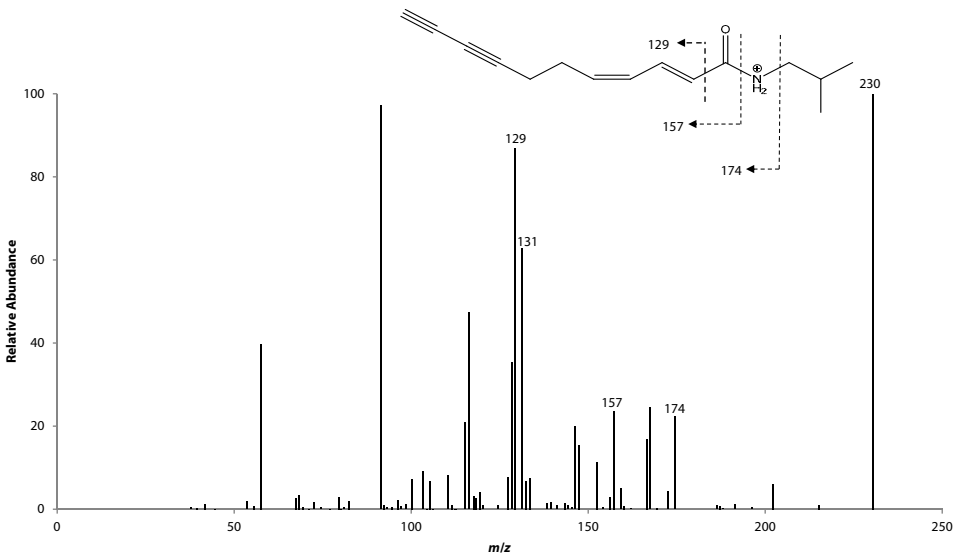

Figure 2. Fragmentation pattern of undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (alkylamide **1**). This product ion spectrum was obtained at t_R = 2.18 min (peak A) after fragmentation of *m/z* 230 $[M + H]^+$ with collision energy -15 V. The presence of the product ions *m/z* 157 (acyllium ion), *m/z* 129 (alkyl chain by loss of isobutylamide), *m/z* 131 (alkyl chain by loss of isobutylamide and saturation double bond), 174 (loss isobutyl group) were used in the identification of this alkylamide.

Peak B

The *m/z* 232 $[M + H]^+$, product ions (*m/z* 91, *m/z* 105 and *m/z* 131) and retention time (t_R = 2.6 min) of the reference standard of undeca-2*E*-ene-8,10-diynoic acid isobutylamide (alkylamide **2**) corresponded with peak B in the extract, thus confirming the presence of this alkylamide in Echinaforce®. This alkylamide was also previously identified and quantified in human plasma after ingestion of Echinaforce® by a human volunteer [9].

Peaks D and G

Alkylamide **4** (dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide, peak D), and alkylamide **8** (dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide, peak G) were identified based on their relative retention time, *m/z* 244 $[M + H]^+$ and fragmentation pattern. The product ion spectrum of peak D and G revealed the following fragments which were reported previously to originate from alkylamides **4** and **8** [2]: *m/z* 143 (alkyl chain after loss of amide portion), *m/z* 145 (alkyl chain after loss of amide portion and saturation double bond), *m/z* 171 (acyllium ion) and *m/z* 188 (protonated alkylamide after loss of the N-isobutyl group).

Based on the previously reported relative elution order (*E*,*Z* isomer after *Z*,*E* isomer) [1, 8], these isomers were distinguished.

Peaks E and H

Peaks E and H were assigned to undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (alkylamide **5**) and undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide (alkylamide **9**), respectively. Retention time, *m/z* $[M + H]^+$ and fragments revealed the identities of these alkylamides. In particular, the following product ions were useful in the identification of these alkylamides: *m/z* 131 (alkyl chain after loss of amide portion and saturation of one the double bonds on the alkyl chain), *m/z* 129 (alkyl chain after loss of amide portion), *m/z* 174 (protonated alkylamide minus the N-2-methylbutyl group) and *m/z* 157 (acyllium ion). Previously reported elution order indicated that the *E*/*Z* isomer elutes earlier than the *Z*/*E* isomer [1, 2].

Peak F

The *m/z* $[M + H]^+$ of 246, retention time and fragmentation pattern of peak F are similar to the data for the reference standard dodeca-2*E*-ene-8,10-diynoic acid isobutylamide (alkylamide **6**), which reveals the presence of this alkylamide. However, co-elution of dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide (alkylamide **7**), which has previously been quantified in an Echinaforce® liquid extract [3], is also possible. This compound has the same *m/z* 246 $[M + H]^+$ and its fragmentation pattern overlaps with that alkylamide **6**. Therefore, the presence of alkylamide **7** in the extract remains uncertain.

Peaks I and J

The identification of this peak as trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide (alkylamide **10**) was based on *m/z* 258 $[M + H]^+$, retention time and the following product ions: *m/z* 157 (alkyl chain after loss of the isobutylamide portion), *m/z* 202 (protonated alkylamide minus the N-isobutylamide group). These product ions of *m/z* 258 $[M + H]^+$ indicated that this alkylamide was an isobutylamide and not dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (alkylamide **11**, *m/z* 258 $[M + H]^+$), which elutes rapidly after peak I.

Peak K

The presence of the product with *m/z* 173 (acyllium ion) reveals that peak K (t_R = 5.6 min) is an 2-methylbutylamide with *m/z* 260 $[M + H]^+$. Therefore, peak K is assigned to dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide (alkylamide **12**). The product ion scan of this peak also showed other products (*m/z* 190, 147, 145, 105, 91) that have been assigned previously to this compound [1].

Peak L and M

Peaks L and M were the most abundant peaks in all tested extracts and they were assigned to the major alkylamides dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides (alkylamides **13** and **14**). As shown in Figure 3 the *m/z* 248 $[M + H]^+$, the product ion spectra and the retention times of these peaks were identical to those of the reference standard of dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides. Accordingly, after ingestion of batch 1 the presence of alkylamides **13** and **14** has previously been shown in patient samples of the Echinaforce® - docetaxel interaction study [10].

Since the two isomers could not be separated under the applied chromatographic conditions, the identification was based on the previously reported elution order (*E*,*E*,*Z*,*Z* isomer before *E*,*E*,*Z*,*E* isomer [1]).

Peak N

MS/MS analysis of peak N (t_R = 7.7 min) indicated that the products of *m/z* 262 $[M + H]^+$ originated from dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide (alkylamide **15**). In particular the product ions *m/z* 175 (acyllium ion), *m/z* 147 (alkyl chain after loss of the 2-methylbutylamide portion) and *m/z* 149 (alkyl chain after loss of the 2-methylbutylamide portion and saturation of one the double bonds on the alkyl chain) were helpful in the identification of alkylamide **15**.

Peak O

Peak O (t_R = 7.8 min) was assigned to dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide (alkylamide **16**). Identification of this alkylamide has been confirmed by the presence

of *m/z* 250 $[M + H]^+$ and the product ions *m/z* 177 (acyllium ion), *m/z* 149 (alkyl chain after loss of the isobutylamide portion), *m/z* 194 (protonated alkylamide after loss of the N-isobutyl group).

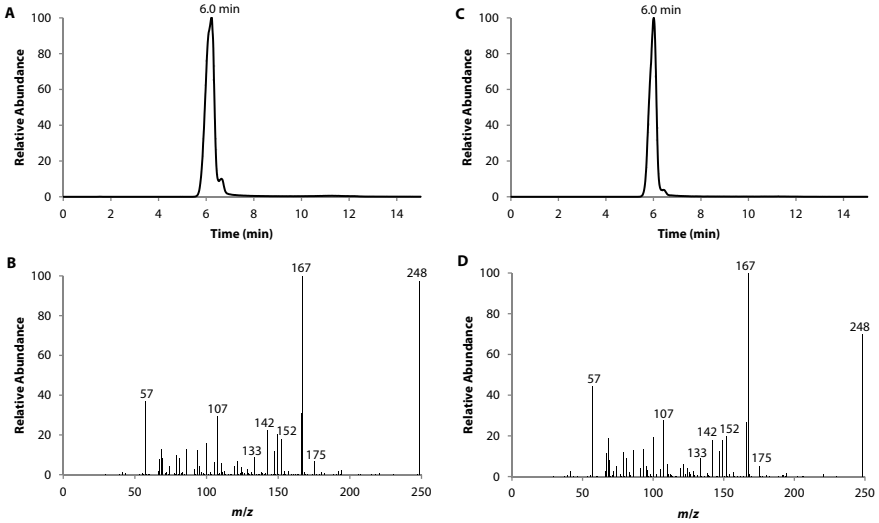

Figure 3. (A) LC-MS/MS chromatogram of the reference standard of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (transition *m/z* 248 $[M + H]^+ \rightarrow$ *m/z* 167, alkylamide **13** and **14**). (B) Product ion spectrum (collision energy -15 V) of the reference standard of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (alkylamide **13** and **14**). The most abundant product ions are annotated. (C) LC-MS/MS chromatogram of peaks L and M (transition *m/z* 248 $[M + H]^+ \rightarrow$ *m/z* 167) in Echinaforce® extract (batch 1). (D) Product ion spectrum (collision energy -15 V) of peaks L and M in Echinaforce® extract (batch 1).
Similar retention times (t_R = 6.0 min), similar *m/z* 248 $[M + H]^+$, and comparable product ion spectra of the reference standard and peaks L and M in the extract indicated the presence of alkylamides **13** and **14** in this batch of Echinaforce®. The most abundant product ions are annotated.

Peak P

Peak P was the single peak in the extracted ion chromatogram of *m/z* 286 $[M + H]^+$. This $[M + H]^+$ and fragments *m/z* 185 (alkyl chain after loss of the isobutylamide portion), *m/z* 230 (protonated alkylamide after loss of the N-isobutyl group) and *m/z* 213 (acyllium ion) after MS/MS analysis corresponded with pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide (alkylamide **17**).

Peak Q

The retention time, *m/z* 252 $[M + H]^+$ and product ion spectrum of the alkylamide eluting at 9.6 min, was identical to that of the reference standard of dodeca-2E,4E-dienoic acid isobutylamide (alkylamide **18**). Therefore, peak Q was assigned to this alkylamide.

In summary, the alkylamides listed in Table 1 were all identified in batch 1. The alkylamides **2**, **6**, **13**/**14**, and **18** could be identified with the highest certainty, as the $[M + H]^+$, retention time and product ion spectra of their reference standards could be used as "fingerprints".

The identification of the other alkylamides was more indirect, since the obtained precursor *m/z* $[M + H]^+$, retention time and fragmentation patterns were compared with data published in literature [1, 2]. Especially the study of Mudge *et al*. [1] was helpful in this regard, since similar chromatographic conditions were applied in this study.

A limitation of this quality control analysis was that without NMR analysis, it was not possible to conclusively distinguish the isomeric configuration of certain alkylamides (alkylamides **1** and **3**, **4** and **8**, **5** and **9**, **13** and **14**) [8]. Therefore, distinction of isomeric pairs was made by previously reported elution order [1, 2]. Further, these isomeric pairs were not completely separated under the applied chromatographic conditions. However, the applied parameters for peak integration were equal for all analyzed batches, implicating that calculated peak areas were well comparable.

Alkylamide content in Echinaforce® batch 1

The previous described identification of alkylamides was used for quantification of each alkylamide in batch 1 which was applied in our clinical interaction study. For the quantification of alkylamides MS/MS analysis was carried out using the SRM transitions listed in Table 3, resulting in a peak area ratio for each alkylamide.
Figure 4 shows that alkylamide **13** and **14** were the most abundant alkylamides in batch 1, followed by alkylamides **17**, **10**, **3** and **1**. This result is in accordance with other studies [1-3, 8] which have shown that alkylamide 13 and 14 were the major alkylamides in *E. purpurea* extracts. Also for Echinaforce® extract in particular, it has previously been shown that the main proportion of alkylamide content was formed by the alkylamides **13**, **14**, **1** and **3** [3].

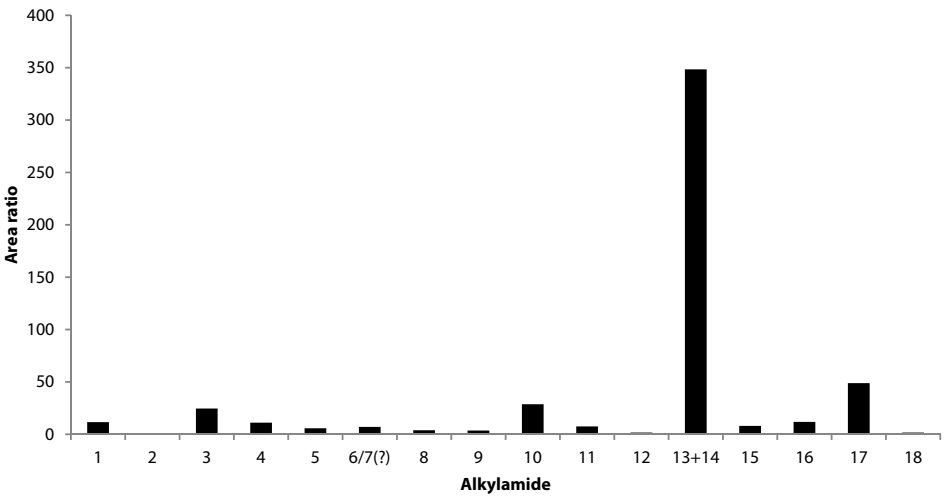

Figure 4. Area ratio (absolute area alkylamide *vs* absolute area internal standard) of assigned alkylamides in Echinaforce® batch 1 after LC-MS/MS analysis.

Comparison of alkylamide content in five Echinaforce® batches

To assess whether the alkylamide content differs between batches of Echinaforce®, five batches were analyzed using MS/MS with the SRM transitions in Table 3. Calculated area ratios were compared with ratios of reference batch 1.

Batch comparison (Figure 5) revealed that, in general, the alkylamide content in batches 2 - 5 did not deviate strongly from the content in batch 1 as indicated by a ratio around 1. Exceptions from this finding were observed for alkylamide 1 and 2. Levels of alkylamide 1 in batch 3 were 2.6-fold higher compared to batch 1. Since the isomeric alkylamides 1 and 3 were shown to be the most potent CYP3A4 inhibitors in *Echinacea* [3], this finding could imply that batch 3 exhibits more potent CYP3A4 inhibition than batch 1. Levels of alkylamide 3 in batch 3, however, were not substantially different from the other tested batches.

Further, levels of the minor alkylamide **2**, which has not been known to affect CYP enzymes, were 3-5 times higher in batches 2-5 compared to batch 1. The similarity in alkylamide content across the five batches suggests that the result of the clinical interaction study between Echinaforce® and docetaxel can be extrapolated to other batches of Echinaforce® liquid extracts. In addition, the CYP-inhibitory properties of the *E. purpurea* batches are presumably also comparable due to the modest inter-batch differences in levels of the CYP-modulating alkylamides **1**, **3**, **13**, **14** and **18**. Alkylamide **13** and **14** are the most abundant alkylamides and have been shown to inhibit CYP2C19, 2D6 and 3A4 [5]. Further, alkylamide **18** has been shown to inhibit CYP2C19, 2D6 and 3A4 [5], while alkylamides **1** and **3** have been reported to exert the most potent inhibition of CYP3A4 [3].

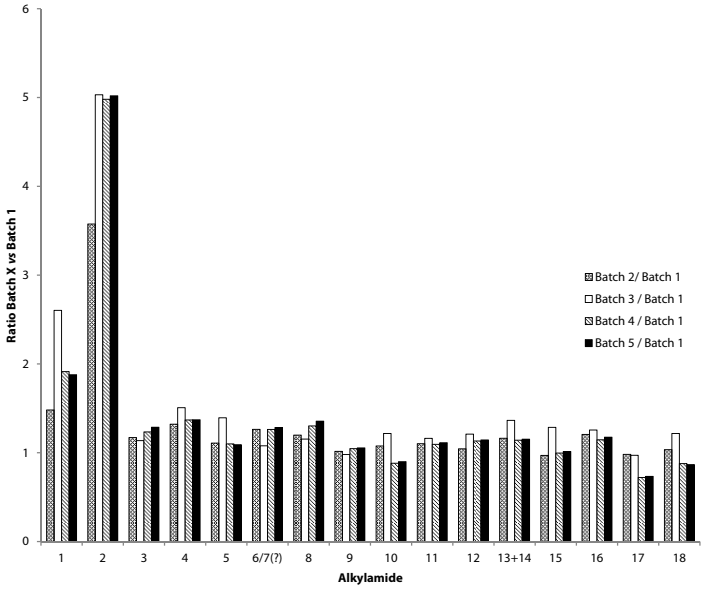

Figure 5. Comparison of alkylamide contents in five Echinaforce® batches. A ratio around 1 indicates no difference in alkylamide content compared to batch 1.

CONCLUSION

In general, herbal medicinal products are complex mixtures of phytochemical compounds and variation of phytochemical content in herbal products is not unusual, as has previously been shown for St. John's wort [11, 12] and *P. ginseng* [13] products. To investigate whether this was also applicable for Echinaforce® liquid extracts, the content of alkylamides was determined and compared among five batches of Echinaforce®.

This study showed that all previously reported alkylamides in *E. purpurea* (Table 1) were identified in the five tested Echinaforce® extracts. Further, the alkylamide content was comparable between the extracts, suggesting that the result of the clinical interaction study between Echinaforce® and docetaxel (submitted for publication) can be generalized for other batches of Echinaforce®.

ACKNOWLEDGEMENTS

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CHAPTER 4

The effect of St. John's wort on docetaxel pharmacokinetics in cancer patients: CYP3A4 induction

CHAPTER 4.1

Pharmacokinetic interaction between St. John's wort and docetaxel in patients with advanced cancer

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ABSTRACT

Background: St. John's wort (SJW, *Hypericum perforatum*) is commonly used by cancer patients. Since its component hyperforin is a known inducer of the cytochrome P450 (CYP) isoenzyme 3A4, concomitant use of SJW can lead to decreased systemic exposure of anticancer drugs metabolized by CYP3A4, possibly resulting in a decreased therapeutic effect. Here, the potential pharmacokinetic interaction between SJW and the sensitive CYP3A4 substrate docetaxel was investigated.

Patients and Methods: Ten evaluable cancer patients were included in this open label, non-randomized, crossover study. The pharmacokinetics of docetaxel (135 mg, IV, 60 min) were compared before (cycle 1) and after fourteen days of supplementation with SJW (three times daily 300 mg extract) in cycle 2. Also docetaxel-related toxicities were evaluated in both cycles.

Results: SJW supplementation resulted in a statistically significant decrease of the mean area under the docetaxel plasma concentration-time curve ($AUC_{0-\infty}$) from 3035 ± 756 ng/mL*h in cycle 1 to 2682 ± 717 ng/mL*h in cycle 2 ($P = 0.045$). Furthermore, the maximum plasma concentration and elimination half-life of docetaxel were (non-significantly) decreased after SJW supplementation. In addition, the incidence of docetaxel-related toxicities was lower after SJW supplementation.

Conclusions: Supplementation of SJW for fourteen days significantly decreased the systemic exposure to docetaxel in cancer patients. Therefore, concomitant use of docetaxel and the applied SJW product (and presumably also other SJW supplements) should be avoided to prevent potential undertreatment of cancer patients.

INTRODUCTION

The herbal antidepressant St. John's wort (SJW, *Hypericum perforatum*) is one of the top-selling herbal supplements in the United States [1]. The popularity of SJW among cancer patients is reflected by its ranking in the top 8 of alternative medicines [2].
Combining SJW with other drugs could cause pharmacokinetic herb-drug interactions. The SJW constituent hyperforin is a well-known activator of the pregnane X receptor (PXR) [3], which results in an increased transcription of the cytochrome P450 3A4 isoenzyme (CYP3A4) and P-glycoprotein (P-gp). Consequently, induction of CYP3A4 and/or P-gp will increase the metabolism and excretion of drug substrates, eventually leading to a decreased systemic exposure and possibly also to a decreased therapeutic efficacy.
As CYP3A4 is involved in the metabolism of many anticancer drugs (e.g. docetaxel, paclitaxel, imatinib, irinotecan), concomitant use of SJW may have serious clinical consequences especially undertreatment. *In vitro* results have already shown that hyperforin increases the metabolism of docetaxel [4]. To date, however, no clinical studies have been executed yet to determine the clinical relevance of this interaction. Based on significant clinical interactions between SJW and the sensitive CYP3A4 substrate midazolam [5-10], and the anticancer drugs imatinib [11, 12] and irinotecan [13], a pharmacokinetic interaction study between SJW and docetaxel should also be performed. Compared to the metabolism of imatinib and irinotecan, in which also enzymes and drug transporters other than CYP3A4 are involved [14, 15], docetaxel is more exclusively metabolized by CYP3A4 [16] and is thus a good substrate to study CYP3A4-mediated pharmacokinetic interactions.
Therefore, this clinical study was designed to determine whether there is a statistically and potentially clinically relevant pharmacokinetic interaction between SJW and docetaxel. The secondary objective was to determine whether the incidence of docetaxel-related toxicities would be affected if a herb-drug interaction would occur.

PATIENTS AND METHODS

Patients

Patients with histological or cytological proof of cancer for whom treatment with docetaxel was considered to be of potential therapeutic benefit, for example patients with advanced breast, gastric, esophagus, bladder, ovarian cancer, non-small cell lung cancer, head and neck cancer and prostate cancer were included. Other inclusion criteria were: age ≥18 years, performance status ≤ 2 according to the World Health Organization (WHO) scale, life expectancy > 3 months, absolute neutrophil count (ANC) ≥ 1.5×10^9/L, platelet count ≥ 100×10^9/L, hemoglobin level ≥ 6.0 mmol/L, hepatic function as defined by serum bilirubin ≤ 1.5 times the upper limit of normal (ULN) and alanine aminotransferase and aspartate

aminotransferase ≤ 2.5 times the ULN, renal function as defined by serum creatinine ≤ 1.5 times ULN or creatinine clearance ≥ 50 mL/min, able and willing to swallow and retain oral medication, to comply to the protocol procedures and to follow dietary restrictions.
Patients were excluded in case of any treatment with investigational drugs within 30 days before the start of the study or the use of herbal supplements within 6 weeks prior to study treatment. Other exclusion criteria were: alcoholism, drug addiction, psychotic disorders leading to non-adequate follow-up, concomitant use of multidrug resistance (MDR) and CYP3A modulating drugs, uncontrolled infectious disease, HIV-1 or HIV-2 type patients, unresolved (> grade 1) toxicities of previous chemotherapy, bowel obstruction or motility disorders that may influence the absorption of drugs, pregnancy, chronic use of H2-receptor antagonists or proton pump inhibitors, neurologic disease that may render a patient at increased risk for peripheral or central neurotoxicity and presence of symptomatic cerebral or leptomeningeal metastases. All patients provided written informed consent prior to study entry and were treated between May 2010 and October 2011.
This study (EudraCT number 2008-000886-41) was approved by the Medical Ethical Committee of the Netherlands Cancer Institute (NKI, Amsterdam, The Netherlands) and conducted in accordance with current standards of Good Clinical Practices, the WHO Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO).

Study design and treatment

This open label, non-randomized, crossover study was conducted at the NKI. On day 1 docetaxel (Taxotere®, Aventis Pharma S.A., Antony Cedex, France) was administered as an absolute dose of 135 mg (60 minutes, IV). The dose of 135 mg was based on a safe dose of 75 mg/m^2 and an average body surface area of 1.8 m^2. From day 7 until the morning of day 22 patients received SJW tablets (Hyperiplant®, 300 mg dry SJW extract standardized to 0.36 – 0.84 mg hypericin and 9 – 19 mg hyperforin, VSM Geneesmiddelen BV, Alkmaar, The Netherlands) at the recommended dose of three times daily one tablet. In the morning of day 22 the second cycle of docetaxel was administered according to the same dosing schedule as day 1 (Figure 1). Standard docetaxel pre-treatment consisted of oral dexamethasone 8 mg twice daily for three consecutive days.

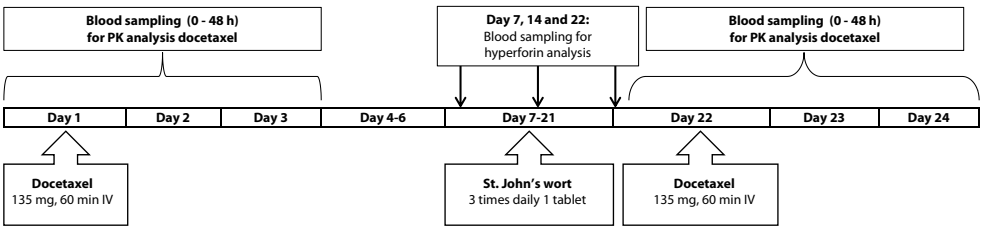

Figure 1. Study design.
On day 1 the first docetaxel cycle was administered. From day 7 until the morning of day 22 SJW was supplemented, followed by a second cycle of docetaxel on day 22. During both docetaxel cycles, blood samples for pharmacokinetic analysis of docetaxel were collected from time 0 until 48 h after start of infusion. On day 7, 14 and 22 blood samples were collected for hyperforin analysis.
IV = intravenous; PK = pharmacokinetic.

Pharmacokinetic analysis for docetaxel

During the two docetaxel cycles blood samples were collected for pharmacokinetic analysis of docetaxel in plasma. Blood samples were drawn at predose, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 7, 10, 24 and 48 h after start of docetaxel infusion. Within 1 hour after collection, samples were centrifuged at 1,500 x *g* for 10 minutes at 4°C, plasma was isolated and stored at -20°C until analysis. Docetaxel plasma concentrations were determined using a validated assay based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [17].

Pharmacokinetic analysis for hyperforin

Adherence of SJW intake by the patients was assessed by checking patients' diaries and measuring the presence of hyperforin in plasma samples obtained at three time points (before start SJW supplementation, seven days after start of SJW and fourteen days after start of SJW).

For analysis of hyperforin an LC-MS/MS assay was used. In short, after addition of 50 µL 5000 ng/mL reserpine (internal standard), 100 µL plasma sample underwent liquid-liquid extraction with 1 mL ethyl acetate-n-hexane (70:30, v/v). After evaporation of the organic phase, the dry extract was reconstituted with 200 µL acetonitrile-water (75:25, v/v) and 20 µL was injected into the LC-MS/MS system.

Liquid chromatography was carried out using a Polaris 3 C18-A column (50 mm x 2 mm ID, particle size 3 µm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 mm x 2 mm ID, particle size 3 µm, Varian). Further, the LC equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler and two LC10-ADvp-µ pumps (all from Shimadzu, Kyoto, Japan). Isocratic elution (25% A, 75% B), delivered at a flow rate of 0.4 mL/min, was applied with mobile phase A consisting of 5 mM ammonium acetate and 0.1% acetic acid in water, while mobile phase B consisted of acetonitrile.

Subsequently, MS/MS analysis on a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with electrospray ionization (ESI) was performed in the negative ion mode. For hyperforin, the mass transitions from *m/z* 535 to 383 were optimized and for the internal standard reserpine, responses from *m/z* 607 to 211 were monitored.
In order to indicate the extent of hyperforin exposure, a calibration curve consisting of three hyperforin concentrations (37.5, 75 and 150 ng/mL) was prepared by spiking hyperforin working solutions (Sigma-Aldrich, St. Louis, MO, USA) to blank human plasma (Sera Laboratories International Ltd., Haywards Heath, UK) measured in duplicate after each set of patient samples. Patient samples were also processed and analyzed in duplicate. Subsequently, the average hyperforin concentrations in these samples were calculated.

Pharmacokinetic and statistical analysis

Based on a statistical power of 80%, a significance level of 5%, an estimated intra-subject standard deviation in docetaxel clearance of 25% and a detectable effect size of 25%, a sample size of 10 evaluable patients was required. This calculation was performed with the Power.T.Test package in *R* version 2.14.0 (*R* Development Core Team, Vienna, Austria).
The following pharmacokinetic parameters of docetaxel were calculated using non-compartmental analysis with *R*: area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$) and maximum plasma concentration (C_{max}).
After logarithmic transformation of these parameters a paired Student's *t* test ($\alpha = 0.05$) was performed using R to detect statistically significant differences between the two treatment cycles.

Docetaxel-related adverse events

Docetaxel-related adverse events during cycle 1 and 2 were registered according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 3.0. Adverse events were considered as docetaxel-related when rated as possibly, probably or definitely related by the investigator.

RESULTS

Baseline patient characteristics

Eleven patients with advanced cancer were included in this study. Patients' baseline characteristics are shown in Table 1. Due to an allergic reaction to docetaxel, one patient did not completely receive the second docetaxel infusion and was therefore not evaluable for pharmacokinetic analysis. Exclusion of this patient resulted in a number of ten patients eligible for the final analysis.

Table 1. Baseline patient characteristics (n = 11)

Gender		
	Female	7
	Male	4
Age (years)		
	Median	62
	Range	40-67
Race		
	Caucasian	11
Primary tumor		
	Bladder	6
	Non-small cell lung carcinoma (NSCLC)	3
	Ovarium	1
	Ureter	1
Previous treatment		
	Chemotherapy	11
	Radiation therapy	4
	Hormonal therapy	1

Pharmacokinetic analysis of docetaxel

Pharmacokinetic parameters of docetaxel before and after SJW intake are shown in Table 2. A paired Student's *t* test revealed that the mean $AUC_{0-\infty}$ of docetaxel significantly decreased from 3035 ± 756 ng/mL*h in cycle1 to 2682 ± 717 ng/mL*h in cycle 2 (P = 0.045). Individual changes in $AUC_{0-\infty}$ are depicted in Figure 2. In eight out of ten patients a decrease of $AUC_{0-\infty}$ was observed. Further, the mean $t_{1/2}$ and C_{max} decreased from 41 ± 35 to 31 ± 10 h (P = 0.185) and from 2000 ± 685 to 1873 ± 710 ng/mL (P = 0.468), respectively. These changes, however, were statistically not significant.

Table 2. Summary of docetaxel pharmacokinetic parameter estimates

Parameter	Day 1	Day 22	*P*
$AUC_{0-\infty}$ (ng/mL*h)	3035 ± 756	2682 ± 717	0.045
C_{max} (ng/mL)	2000 ± 685	1873 ± 710	0.468
$t_{1/2}$ (h)	41 ± 35	31 ± 10	0.185

Pharmacokinetic data are given as mean ± SD and were obtained on day 1 (docetaxel alone) and day 22 (14 days after start of St. John's wort). *P* values were obtained from a paired Student's *t* test.
Abbreviations: $AUC_{0-\infty}$ = area under the docetaxel plasma concentration - time curve extrapolated to infinity; C_{max} = maximum plasma concentration; $t_{1/2}$ = elimination half-life.

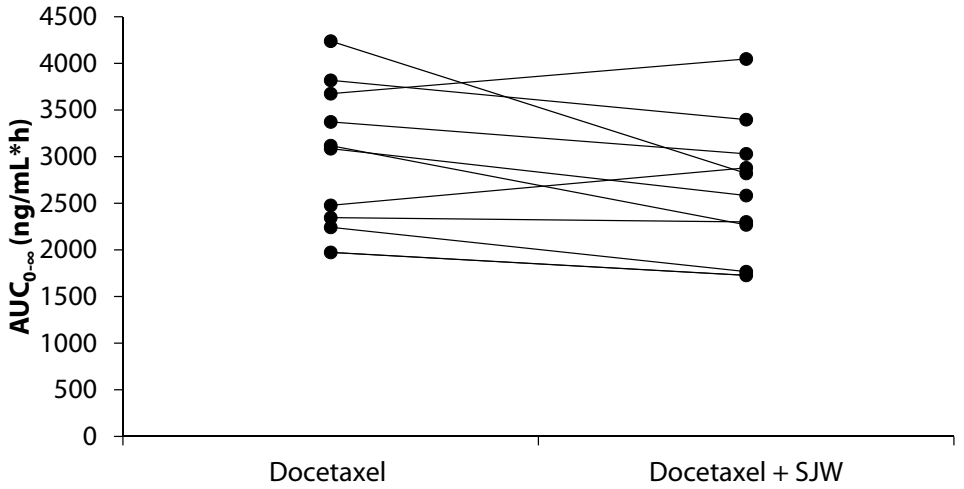

Figure 2. Comparison of individual docetaxel $AUC_{0-\infty}$ values obtained before St. John's wort administration and 14 days after St. John's wort intake (n = 10).
$AUC_{0-\infty}$ = area under the docetaxel plasma concentration-time curve extrapolated to infinity; SJW = St. John's wort.

Hyperforin analysis in plasma

As expected, before start of SJW supplementation no hyperforin was detected in the majority of the patients (Table 3). In one patient, however, a relatively high hyperforin concentration (> 75 ng/mL) was found, which was caused by the fact that the first tablet of SJW was accidentally ingested *before* baseline PK blood sampling. As this patient ingested all other SJW doses according to protocol, this patient was considered evaluable for pharmacokinetic analysis.

After seven and fourteen days of SJW intake, hyperforin was detected in all patient samples. In addition to the patients' diaries, this result confirmed that all patients were compliant to SJW intake.

Concerning the relationship between measured hyperforin levels and the extent of CYP3A4 induction, it is worthwhile noticing that the two patients with the highest hyperforin levels on day 1 of cycle 2, also showed the greatest decrease in $AUC_{0-\infty}$ of docetaxel (data not shown). For the other patients, however, no apparent correlation between hyperforin levels and docetaxel $AUC_{0-\infty}$ was observed.

Table 3. Hyperforin plasma concentrations obtained at predose (day 7) and seven days (day 14) and fourteen days (day 22) after start of St. John's wort supplementation

Mean hyperforin plasma concentration (ng/mL)	**Day 7 (predose)** No. patients	**Day 14** No. patients	**Day 22** No. patients
< 37.5	9*	2	6
37.5-75	0	5	4
> 75	1	3	0
* Not detectable			

Docetaxel-related adverse events

The incidence of docetaxel-related toxicities according to NCI CTCAE version 3.0 are displayed in Table 4.

Table 4. Incidence of docetaxel-related adverse events (NCI CTCAE version 3.0)

	Cycle 1 (without SJW, *n* = 10)		**Cycle 2 (with SJW, *n* = 10)**	
	Grade 1-2	**Grade 3-4**	**Grade 1-2**	**Grade 3-4**
Patients with adverse events, *n*	8	1	8	0
Adverse events, *n*				
Fatigue	7	-	4	-
Myalgia	4	-	3	-
Alopecia	5	-	6	-
Nausea	3	-	3	-
Fever	3	-	3	-
Allergic reaction	1	-	2	-
Constipation	3	-	-	-
Pain (bone)	1	-	1	-
AST	2	-	-	-
ALT	-	1	-	-
Anemia	-	-	1	-
Occular (irritation)	-	-	1	-
Pain (ear)	1	-	-	-
Dry mouth	1	-	-	-
Pain (throat)	1	-	-	-
Neuropathy (fingers)	1	-	-	-
Watery eye (right)	1	-	1	-
Tinnitus	1	-	-	-
Stomatitis	1	-	-	-
Rash/pruritus	1	-	-	-
Diarrhea	1	-	-	-
Total adverse events, *n*	**38**	**1**	**25**	**0**

Nine out of ten patients suffered from docetaxel-related adverse events, which were mainly grade 1-2 toxicities. The most common adverse events were fatigue, myalgia, alopecia, nausea and fever. Overall, the incidence of docetaxel-related adverse events was lower after SJW supplementation (25 adverse events) than before (39 adverse events). In

50% of the patients the decrease in $AUC_{0-\infty}$ was accompanied by a decrease in docetaxel-related adverse events and also a decrease in co-medication use (data not shown).
Objective toxicities such as elevated serum levels of aspartate transaminase (AST), alanine transaminase (ALT) and anemia occurred more frequently in cycle 1 (3 adverse events) than in cycle 2 (1 adverse event), however as this concerns low numbers in a small population these data cannot be further interpreted.

DISCUSSION

The present study was intended to investigate whether supplementation of SJW affects the pharmacokinetics of docetaxel. SJW has already been shown to be a potent CYP3A4 inducer in *in vitro* [3, 18, 19] and clinical studies [5-10], thus an increased hepatic clearance and reduced systemic exposure to docetaxel was expected. Pharmacokinetic analysis showed that the results were in concordance with this hypothesis. Supplementation of the commercial SJW product Hyperiplant® dosed as three times daily one tablet for two weeks, resulted in a reduction in exposure to docetaxel. The $AUC_{0-\infty}$ of docetaxel decreased by 12% on average, which was statistically ($P < 0.05$) significant. Furthermore, $t_{1/2}$ and C_{max} decreased by 24% and 6% after SJW supplementation. Although these changes were statistically not significant, they were in line with an increased elimination of docetaxel. Hyperforin levels that significantly induced the metabolism of docetaxel via CYP3A4 *in vitro* (~ 50 ng/mL) [4], were also reached in the majority of the patients in this study (Table 2). This finding indicates that induction of CYP3A4 and docetaxel metabolism by hyperforin was highly likely in the current study.
The observed decrease in $AUC_{0-\infty}$ of docetaxel after SJW supplementation could affect the therapeutic effect of docetaxel, since systemic exposure to docetaxel is directly related to its efficacy and toxicity [20]. Cancer patients receiving docetaxel and SJW could thus be at risk of undertreatment.

In addition to the clinical interaction studies combining SJW with imatinib [11, 12] and irinotecan [13], our study is the fourth study investigating the pharmacokinetic interaction between SJW and an anticancer drug. In comparison with the imatinib and irinotecan interaction studies SJW had a smaller effect on the pharmacokinetics of docetaxel in the present study. For example, the AUC of irinotecan's active metabolite SN-38 significantly decreased by 42% after supplementation of SJW [13] and the $AUC_{0-\infty}$ of imatinib was reduced by 30 - 32% [11, 12]. Compared to imatinib, the less evident 12% decrease of the $AUC_{0-\infty}$ of docetaxel in the present study, can be explained by differences in route of administration. Imatinib is administered orally, thus additional induction of intestinal CYP3A4 and P-gp may have increased the potency of the interaction. It has already been shown that

hyperforin has more potent CYP3A4 inducing effects in the intestinal wall than in the liver [5, 6, 9, 21]. Consequently, administration of SJW is expected to have a more potent effect on the pharmacokinetics of orally administered CYP3A4 substrates.
Regarding irinotecan, SJW may have affected carboxylesterases [22] and uridine diphosphate glucuronosyltransferases 1A (UGT1A) [23] which are, in contrast to CYP3A4, directly involved in the formation and metabolism of the active metabolite SN-38. Thus besides CYP3A4 induction SJW could affect the pharmacokinetics of irinotecan in more ways than docetaxel, which is mainly metabolized by CYP3A4.

In the present interaction study patients were not randomized for sequence of treatment, which may be considered as a limitation. The absence of randomization, however, is not expected to introduce substantial bias to the pharmacokinetic endpoints of this study.
First, based on literature data also no significant period effect of docetaxel exposure is expected [24, 25]. Pharmacokinetic studies have shown that the intrapatient variability of the AUC_{0-24} of docetaxel was minor (mean ratio cycle 2 to cycle 1 was 1.11 ± 0.14) after repeated administration of docetaxel administered over 1 h at a dose of dose 55 mg/m^2 every three weeks [25]. In accordance to this finding also Brunsvig *et al*. reported a similar intrapatient variability after repeated 3-weekly administration of docetaxel dosed at 100 mg/m^2 over 1 h [24]: the median ratio of the AUC_{0-25} of docetaxel in cycle 2 to cycle 1 was 1.13 (range 0.9 - 1.5). Furthermore, the pharmacokinetic endpoints in the present study are objective outcomes, thus biased results by learning effects are very unlikely.
Second, the absence of docetaxel in the predose plasma samples of cycle 2 showed that there was no carry-over effect, thus the washout period of three weeks was adequate.
Third, the bioanalytical assay for docetaxel was well validated and for every single patient the plasma samples of both cycles were analyzed within the same analytical run. Therefore, the analytical results were not likely to be affected by the sequence of treatment.

It should be noted that the presented results specifically apply to the SJW product Hyperiplant®. It cannot be excluded that other SJW products differ in their potential to affect the pharmacokinetics of docetaxel. The SJW product used in the present study has been approved as a drug by the Dutch Medicines Evaluation Board. Consequently, the contents are strictly standardized to contain 0.36 to 0.84 mg hypericin and 9 to 18 mg hyperforin. In contrast, the composition of other commercially available non-registered SJW products is not always clear. Further, it has already been shown that different brands of SJW products differ in their contents of hyperforin and hypericin [26-28]. With this knowledge and the fact that the extent of CYP3A4 induction depends on hyperforin content [29], the severity of pharmacokinetic interactions between docetaxel and other SJW products is fairly unpredictable and can vary drastically.

In conclusion, the results of the present clinical study showed that the medicinal SJW product Hyperiplant® at the recommended dose of three times daily one tablet, significantly decreased the systemic exposure to docetaxel. In order to prevent potential undertreatment of cancer patients using docetaxel, it is recommended to avoid concomitant use of this SJW product and docetaxel. Considering the interproduct variability in hyperforin content it is also advisable not to combine SJW products and docetaxel in general.

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4

CHAPTER 5

The effect of milk thistle on the pharmacokinetics of docetaxel and tolbutamide in cancer patients: CYP2C9 and CYP3A4 inhibition

CHAPTER 5.1

The effect of milk thistle on the pharmacokinetics of docetaxel and tolbutamide

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Interim analysis: preliminary data of ongoing study

ABSTRACT

Background: Milk thistle (*Silybum marianum*) is a popular herbal supplement among cancer patients. Concomitant use of milk thistle, however, may interfere with conventional anticancer drugs due to potential pharmacokinetic interactions. *In vitro* and clinical studies suggest that milk thistle inhibits the cytochrome P450 (CYP) isoenzymes 2C9 and 3A4. This clinical study investigates the effect of a highly bioavailable milk thistle formulation on the pharmacokinetics of tolbutamide and docetaxel, sensitive substrates for CYP2C9 and CYP3A4, respectively. Since this study is ongoing, preliminary results are presented in this interim analysis.

Patients and Methods: At the time of this analysis two patients with advanced cancer were evaluable in this open label, randomized, crossover study, which in the end requires ten evaluable patients. The pharmacokinetics of oral tolbutamide (250 mg) and intravenous docetaxel (135 mg, 2 h infusion) were compared before and after a 4-day supplementation period with milk thistle capsules (3 times daily 180 mg silibinin-phosphatidylcholine). Docetaxel-related toxicities were also evaluated.

Results: In both patients alterations in the plasma concentration-time curves of docetaxel, tolbutamide and its CYP2C9-mediated metabolite 4-hydroxytolbutamide did not indicate inhibition of CYP2C9 and CYP3A4. In both patients the area under the plasma concentration-time curves ($AUC_{0-\infty}$) and maximum plasma concentrations (C_{max}) of docetaxel hardly changed (approximately 5% difference) before and after milk thistle supplementation. Accordingly, the incidence of docetaxel-related adverse events in these patients was comparable across the two treatment cycles. Changes in the pharmacokinetics of tolbutamide and 4-hydroxytolbutamide, were also modest (e.g. ≤ 11% difference in $AUC_{0-\infty}$) and inconsistent with CYP2C9 inhibition.

Conclusions: These preliminary data indicate that the applied milk thistle product did not substantially affect the metabolism of docetaxel and tolbutamide in two patients. However, conclusions about the statistical and clinical relevance of the obtained results cannot be drawn prior to evaluation of the last patient.

INTRODUCTION

Milk thistle (*Silybum marianum*) is a widely used herbal supplement [1]. Silymarin, extracted from milk thistle seeds, is used for treatment of liver disorders (including hepatic drug toxicities) due to its claimed hepatoprotective and detoxifying properties. Other reported indications are *Amanita phalloides* mushroom poisoning, diabetes and hyperlipidemia. Silymarin is a complex consisting of one flavonoid and seven flavonolignans. The flavonolignans silybin A and silybin B, which form the isomeric mixture silibinin (1:1), are the most abundant constituents [2]. Also among cancer patients the use of milk thistle supplements is popular [1]. However, concomitant use of milk thistle and anticancer drugs may lead to undesirable effects due to pharmacokinetic interactions. *In vitro* studies have reported that milk thistle extract and its components silymarin and silibinin inhibit the cytochrome P450 (CYP) enzymes 2C9 [3, 4] and 3A4 [4, 5]. In addition, inhibition of these CYP enzymes by milk thistle extract has been confirmed in our laboratory (data not shown). Inhibition of CYP2C9 and CYP3A4 by silibinin has also been suggested in rats, since the biotransformation from tamoxifen to 4-hydroxytamoxifen, involving among others CYP2C9 and CYP3A4 [6], was inhibited after silibinin administration [7]. In accordance to the observed inhibition of CYP2C9 and CYP3A4 by milk thistle *in vitro*, silymarin significantly inhibited the metabolism of losartan in healthy volunteers [8]. However, the underlying mechanism of this interaction remains uncertain, since both CYP2C9 and CYP3A4 are involved in the metabolism of losartan.

CYP2C9 and CYP3A4 are also involved in the metabolism of many other anticancer drugs. Since anticancer drugs have narrow therapeutic windows, alterations of their plasma concentrations due to CYP inhibition by milk thistle, could have serious consequences, such as undertreatment or adverse effects. Cyclophosphamide, ifosfamide, tamoxifen and tegafur are metabolized by CYP2C9. CYP3A4 metabolizes a wide range of anticancer drugs including docetaxel, irinotecan, imatinib, tamoxifen and etoposide [9].

However, previous clinical studies have shown that milk thistle did not significantly affect the pharmacokinetics of midazolam [10, 11] and irinotecan [12]. The lack of pharmacokinetic interactions in these studies was partially attributed to poor bioavailability of milk thistle. Therefore, this study was set up to investigate the clinical effect of a highly bioavailable milk thistle formulation on the pharmacokinetics of docetaxel, which is a more sensitive substrate for CYP3A4 than irinotecan. In this study a silibinin-phosphatidylcholine complexed product was applied, as phosphatidylcholine has previously been shown to significantly enhance the bioavailability of silibinin [13] Thus, the combined use of docetaxel as a CYP3A4 substrate and a milk thistle preparation with enhanced bioavailability may lead to a significant CYP3A4-mediated interaction.

Further, the inhibitory effects of milk thistle on CYP2C9 have not been studied previously in humans. Therefore, the sensitive CYP2C9 substrate tolbutamide was coadministered in this study to investigate whether milk thistle would affect plasma levels of tolbutamide and its CYP2C9-mediated metabolite 4-hydroxytolbutamide.

In summary, the primary aim of the present study was to investigate the effect of a highly bioavailable milk thistle product on the pharmacokinetics of docetaxel and tolbutamide in patients with advanced cancer. Second, the incidence of docetaxel- and tolbutamide-related toxicities was monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE), version 4.02. As the clinical study is still ongoing, this interim analysis shows the partially monitored results of the first two evaluable patients.

PATIENTS AND METHODS

Patients

Patients with histological or cytological proof of cancer for whom treatment with docetaxel was considered to be of potential therapeutic benefit were included. This includes patients with advanced breast, gastric, esophagus, bladder, ovarian cancer, non-small cell lung cancer, head and neck cancer and prostate cancer. Other inclusion criteria were: age ≥18 years, performance status ≤ 2 according to the World Health Organization (WHO) scale, life expectancy > 3 months, absolute neutrophil count (ANC) ≥ 1.5×10^9/L, platelet count ≥ 100×10^9/L, hemoglobin level ≥ 6.0 mmol/L, hepatic function as defined by serum bilirubin ≤ 1.5 times the upper limit of normal (ULN) and alanine aminotransferase and aspartate aminotransferase ≤ 2.5 times the ULN, renal function as defined by serum creatinine ≤ 1.5 times ULN or creatinine clearance ≥ 50 mL/min, able and willing to swallow and retain oral medication, and to comply to the protocol procedures and to follow dietary restrictions.
Patients were excluded in case of any treatment with investigational drugs within 30 days before the start of the study, or the use of herbal supplements within 6 weeks prior to study treatment. Other exclusion criteria were: alcoholism, drug addiction, psychotic disorders leading to non-adequate follow-up, concomitant use of multidrug resistance (MDR), CYP2C9 and CYP3A modulating drugs, type I and II diabetes mellitus, uncontrolled infectious disease, HIV-1 or HIV-2 type patients, unresolved (> grade 1) toxicities of previous chemotherapy, bowel obstruction or motility disorders that may influence the absorption of drugs, pregnancy, chronic use of H2-receptor antagonists or proton pump inhibitors, neurologic disease that may render a patient at increased risk for peripheral or central neurotoxicity and presence of symptomatic cerebral or leptomeningeal metastases. The patients in this interim analysis provided written informed consent prior to study entry

and were treated between March 2012 and December 2012.
The current study (The Netherlands Trial Registry: NTR3611) was approved by the Medical Ethical Committee of the Netherlands Cancer Institute (Amsterdam, The Netherlands) and conducted in accordance with current standards of Good Clinical Practices, the WHO Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO).

Study design and treatment

The present phase I study used a randomized, crossover, open-label design (Figure 1). Ten patients were randomized to cohort A or B, each consisting of two cycles.
For cohort A, milk thistle capsules (Siliphos®, 180 mg silibinin-phosphatidylcholine complex, batch 304090 Thorne Research Inc., Dover, USA) were dosed as three times daily one capsule from day 0 until the morning of day 3. Compliance of milk thistle intake was checked by capsule count in returned containers and inspection of diaries in which patients needed to register the times of milk thistle intake. In the morning of day 1, 250 mg of oral tolbutamide (Teva Pharmachemie, Haarlem, The Netherlands) followed by 135 mg of intravenous (2 h) docetaxel (Taxotere®, Aventis Pharma S.A., Antony Cedex, France) was administered. After a 3-week washout period, patients received their second cycle of intravenous docetaxel (135 mg, 2 h) and 250 mg of oral tolbutamide on day 22.
For cohort B, the study started on day 1 with 135 mg of oral tolbutamide and intravenous administration of docetaxel (135 mg, 2 h). From day 21 until the morning of day 24, milk thistle was supplemented (three times daily one capsule). On day 22 docetaxel and tolbutamide were administered according to the same schedule as on day 1.
For both cohorts, the study ended three weeks after the second administration of docetaxel and tolbutamide with an end of treatment visit.

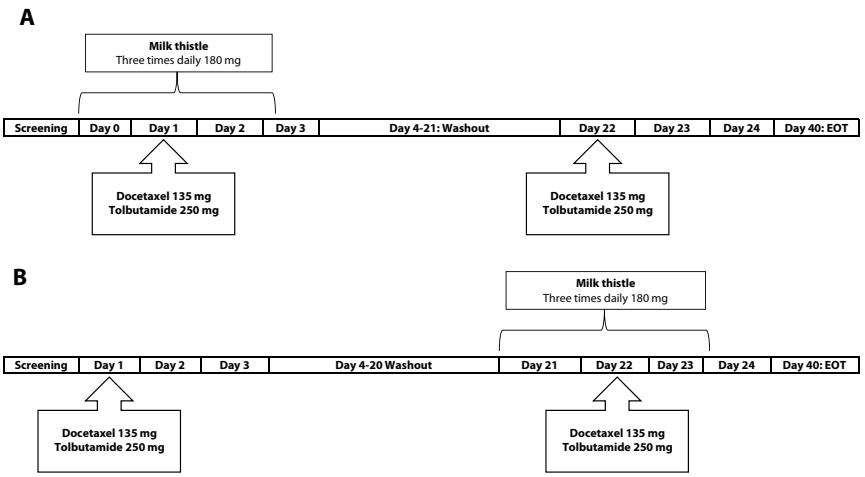

Figure 1. (A) Study design cohort A. (B) Study design cohort B.
Last intake of milk thistle was in the morning of day 3 (cohort A) or day 24 (cohort B).
EOT = End of treatment visit.

Pharmacokinetic analysis

On day 1 and 22 blood samples were collected for pharmacokinetic analysis of docetaxel and tolbutamide. Sampling times for docetaxel samples were 0, 0.5, 1, 1.5, 2, 2.5, 3, 5, 8, 11, 24 and 48 h after start of infusion. For tolbutamide analysis, blood samples were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 9, 12, 25 and 49 h after oral intake.

Within 1 hour after collection, samples were centrifuged at 1,500 x *g* for 10 minutes at 4°C, plasma was isolated and stored at -20°C until analysis. Docetaxel plasma concentrations were determined using a validated assay based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [14]. Plasma concentrations of tolbutamide and its CYP2C9-mediated metabolite 4-hydroxytolbutamide were also determined by an LC-MS/MS assay, which has been validated over a concentration range of 50 - 50,000 ng/mL for tolbutamide and 5 - 5,000 ng/mL for 4-hydroxytolbutamide according to the FDA Guidelines on Bioanalytical Method Validation [15]. Validated parameters, such as between-day inaccuracy (± 8.58%), within-day precision (CV ≤ 8.79%) and between-day precision (CV ≤ 10.86%), fell within the accepted ranges for both tolbutamide and 4-hydroxytolbutamide. Also no interference of endogenous plasma components or co-medication (docetaxel, dexamethasone and silibinin) was observed. In this assay 50 µL plasma samples were prepared by adding 10 µL of internal standard solution (10,000 ng/mL tolbutamide-d9 and 4-hydroxytolbutamide-d9 in methanol-water (50:50, v/v)). Subsequently, 100 µL acetonitrile was added and samples were vortex-mixed and centrifuged for 10 min at 13,500 x *g* (4°C). The clear supernatant was then evaporated with nitrogen at 40°C and reconstituted with 100 µL of 0.2% formic acid in water-methanol-acetonitrile (75:12.5:12.5, v/v). After centrifuging for 10 min at 13,500 x *g* (4°C), 20 µL of the clear supernatant was injected into the LC-MS/MS system. The LC-MS/MS system consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-µ pumps (all from Shimadzu, Kyoto, Japan), and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with electrospray ionization (ESI) which operated in the positive ion mode with both quadrupoles set at 0.7 full width at half maximum (FWHM, unit resolution) and with dwell times of 200 ms. Injections were made on a Polaris 3 C18-A column (50 x 2 mm ID, particle size 3 µm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 x 2 mm ID, particle size 3 µm, Varian). The column temperature was maintained at 40°C and the autosampler was set at 4°C.

Liquid chromatography was carried out using 0.2% formic acid in water (mobile phase A) and methanol-acetonitrile (50:50, v/v, mobile phase B) delivered at a flow rate of 0.5 mL/min. During the first 2.5 min of the run 25% mobile phase B was pumped over the column. From 2.5 until 3.5 min the proportion of mobile phase B increased from 25 to 100%, after which % B was kept constant at 100% for until 4.0 min. Thereafter, the column was reconditioned at the starting conditions for 2 min. For tolbutamide, tolbutamide-d9,

4-hydroxytolbutamide and 4-hydroxytolbutamide-d9 the mass transitions from *m/z* 271 to 91 (collision energy -30 V), 280 to 91 (collision energy -30 V), 287 to 171 (collision energy -15 V) and 296 to 171 (collision energy -15 V) were monitored, respectively.
For data acquisition and processing, Xcalibur software (version 1.4, Thermo Fisher Scientific) was used.

Adverse events

Docetaxel-related adverse events during cycle 1 and 2 were registered according to NCI CTCAE version 4.02. Adverse events were considered as docetaxel- or tolbutamide-related when rated as possibly, probably or definitively related by the investigator. Tolbutamide-related adverse events were not expected, since a subtherapeutic dose of 250 mg was administered. Nevertheless patients' blood glucose levels were measured at 2h after intake of tolbutamide to register hypoglycemic events.

Pharmacogenetic analysis

For genotyping of the polymorphic CYP2C9 enzyme, 4 mL blood was collected on day 1 and stored at -20°C. After inclusion of the last evaluable patient these samples will be analyzed with a TaqMan assay to detect CYP2C9 poor metabolizers with CYP2C9*2 and CYP2C9*3 variant alleles.

Statistical analysis

Based on a statistical power of 80%, a significance level of 5%, an estimated intra-subject standard deviation in docetaxel clearance of 25% and a detectable effect size of 25%, a sample size of 10 evaluable patients was required. This calculation was performed with the Power.T.Test package in *R* version 2.14.0 (*R* Development Core Team, Vienna, Austria).
Using non-compartmental analysis with *R* software the following pharmacokinetic parameters of docetaxel and tolbutamide were calculated: area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), elimination half-life ($t_{1/2}$) and maximum plasma concentration (C_{max}).
After the end of treatment visit of the last evaluable patient, a paired Student's *t* test ($\alpha = 0.05$) on the log-transformed pharmacokinetic parameters will be performed to detect statistically significant differences between the two treatment cycles.
This interim analysis only shows pharmacokinetic parameters of docetaxel and tolbutamide. Due to insufficient statistical power statistical analysis has not been performed on the limited number of evaluable patients thus far.

RESULTS

Patients

At the time of this interim analysis, six patients were included. In Table 1 the patients' baseline characteristics are depicted. Three patients completed cycle 1, but did not receive cycle 2 due to patient refusal (n = 1) and deteriorated medical condition (n = 2). Further, the physical condition of one patient did not allow him to start with study treatment and was therefore withdrawn from the study after the baseline visit. After exclusion of these four patients, a total number of two patients ("patient 1" and "patient 2") completed both treatment cycles and were thus evaluable. Patient 1 and 2 were randomized to cohort B and A, respectively. Patient 1 suffered from grade 4 neutropenia in cycle 1 and therefore the dose of docetaxel was reduced with 25% from 135 mg to 100 mg in cycle 2. For the pharmacokinetic analyses of docetaxel the calculated pharmacokinetic parameters of cycle 2 can be adjusted accordingly for this patient, since docetaxel exhibits linear pharmacokinetics [16].

Table 1. Baseline patient characteristics (n = 6)

Gender		
	Female	1
	Male	5
Age (years)		
	Median	58
	Range	36-66
Race		
	Caucasian	6
Primary tumor		
	Bladder	1
	Non-small cell lung carcinoma (NSCLC)	1
	Small cell lung carcinoma (SCLC)	1
	Gastric	1
	Nasopharynx	1
	Esophageal	1
Previous treatment		
	Chemotherapy	6
	Radiation therapy	4
	Surgery	3

Effect of milk thistle on the pharmacokinetics of docetaxel

Figure 2A and 2B show the plasma concentration-time curves of docetaxel in the two evaluable patients. The curve of patient 1 shows that the docetaxel plasma concentrations were decreased after milk thistle supplementation. In accordance, both the $AUC_{0-\infty}$ and C_{max} were lowered by 22.2% and 22.9%, respectively (Table 2). These changes are most likely caused by the docetaxel dose reduction of 25% in this patient due to grade 4 neutropenia. The $t_{1/2}$ remained fairly constant I in this patient.

The concentration-time curves of patient 2 (Figure 2B) indicate that the systemic exposure to docetaxel was comparable before and after supplementation of milk thistle. In line with

the curves in Figure 2B, the $AUC_{0-\infty}$ and C_{max} were comparable between the two cycles (Table 2).

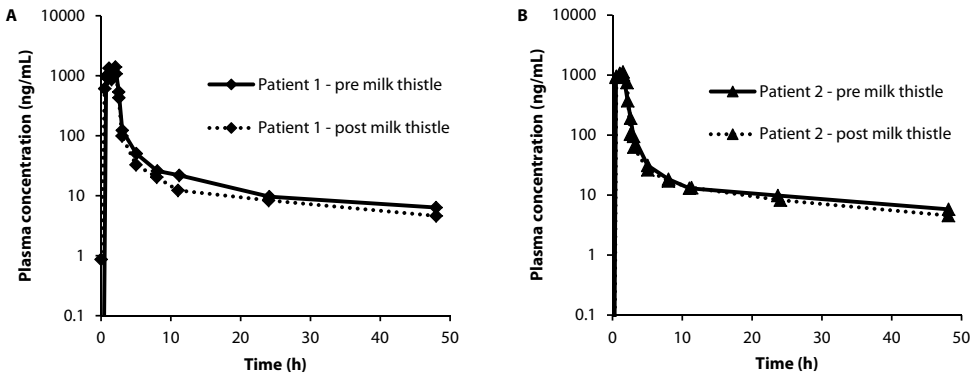

Figure 2. (A) Plasma concentration-time curves for docetaxel in patient 1 before and after milk thistle supplementation. (B) Plasma concentration-time curves for docetaxel in patient 2 before and after milk thistle supplementation.

Table 2. Individual pharmacokinetic parameters for docetaxel

	Pre milk thistle	Post milk thistle	% difference (post *vs* pre)
$AUC_{0-\infty}$ patient 1 (ng/mL*h)	3401	3573* (2647)	5.1 (-22.2)
$AUC_{0-\infty}$ patient 2 (ng/mL*h)	2643	2563	-3.0
C_{max} (ng/mL) patient 1	1400	1458 (1080)**	4.1 (-22.9)
C_{max} (ng/mL) patient 2	1130	1070	-5.3
$t_{1/2}$ (h) patient 1	19.8	21.1	6.5
$t_{1/2}$ (h) patient 2	26.7	21.9	-18.0

$AUC_{0-\infty}$ = area under the plasma concentration-time curve extrapolated to infinity; C_{max} = maximum plasma concentration; $t_{1/2}$ = elimination half-life.
* $AUC_{0-\infty}$ normalized to 100% docetaxel dose by multiplying the measured $AUC_{0-\infty}$ with correction factor 1.35 (135 mg/100 mg). Value between brackets represents the measured $AUC_{0-\infty}$, which was 22.2% lower compared to the $AUC_{0-\infty}$ in cycle 1.
** C_{max} normalized to 100% docetaxel dose by multiplying the measured C_{max} with correction factor 1.35 (135 mg/100 mg). Value between brackets represents the measured C_{max}, which was 22.9% lower compared to the C_{max} in cycle 1.

Effect of milk thistle on the pharmacokinetics of tolbutamide

The individual plasma concentration-time curves of tolbutamide and 4-hydroxytolbutamide before and after milk thistle supplementation are depicted in Figure 3 and Figure 4, respectively. Considering the overlapping curves of both patients, supplementation of milk thistle does not seem to significantly affect the metabolism of tolbutamide. In accordance to the measured plasma concentrations of tolbutamide and 4-hydroxytolbutamide, the pharmacokinetic parameters of these compounds (Table 3) do also not indicate CYP2C9 inhibition.

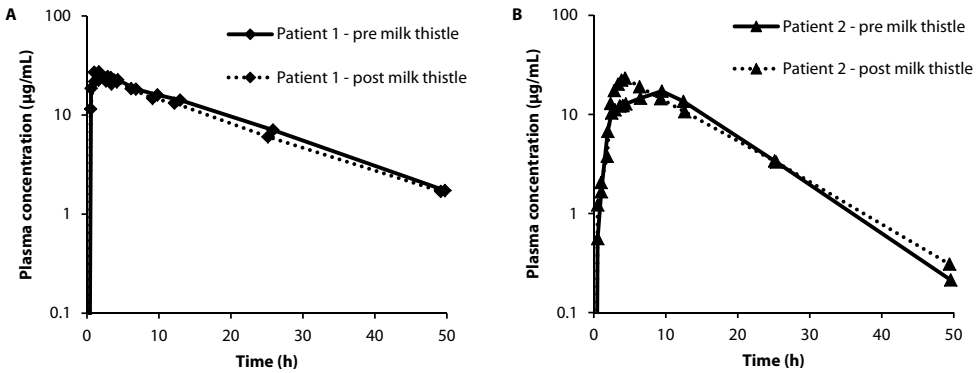

Figure 3. (A) Plasma concentration-time curves for tolbutamide in patient 1 before and after milk thistle supplementation. (B) Plasma concentration-time curves for tolbutamide in patient 2 before and after milk thistle supplementation.

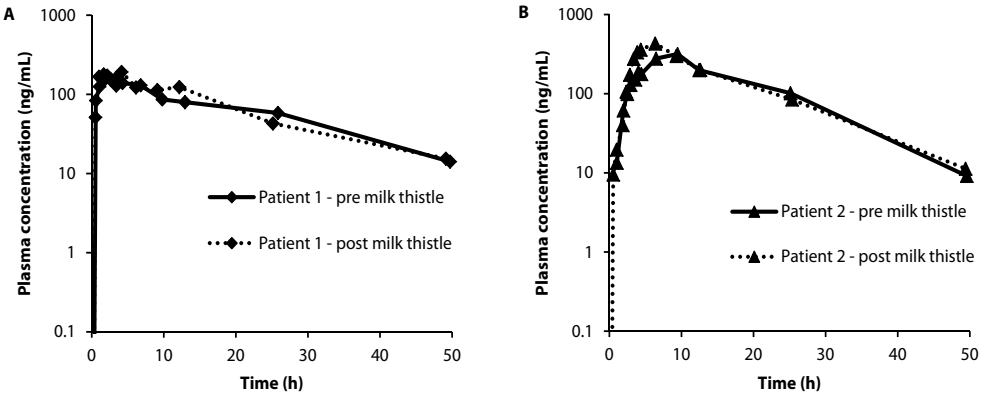

Figure 4. (A) Plasma concentration-time curves for 4-hydroxytolbutamide in patient 1 before and after milk thistle supplementation. (B) Plasma concentration-time curves for 4-hydroxytolbutamide in patient 2 before and after milk thistle supplementation.

In patient 1 changes in tolbutamide pharmacokinetics were minor (Table 3) and did not reflect decreased metabolism of tolbutamide, in which case the $AUC_{0-\infty}$ and C_{max} should increase. Here, however, these parameters changed in opposite directions. Further, the $AUC_{0-\infty}$ of 4-hydroxytolbutamide remained nearly constant (4.4% difference between cycles) which is also conflicting with CYP2C9 inhibition.

In patient 2 the $AUC_{0-\infty}$, C_{max} and $t_{1/2}$ of tolbutamide were all increased after milk thistle supplementation with 5.5%, 35.3% and 15.0%, respectively. The significance of these findings is unknown, but these changes are in line with CYP2C9 inhibition. The pharmacokinetic data of 4-hydroxytolbutamide, however, conflicted with CYP2C9 inhibition, since the $AUC_{0-\infty}$ (11.0%), C_{max} (35.8%) and $t_{1/2}$ (7.1%) of this metabolite were increased.

Table 3. Individual pharmacokinetic parameters for tolbutamide and 4-hydroxytolbutamide

	Pre milk thistle	Post milk thistle	% difference (post vs pre)
Tolbutamide			
$AUC_{0-\infty}$ patient 1 (µg/mL*h)	466	421	-9.5
$AUC_{0-\infty}$ patient 2 (µg/mL*h)	274	289	5.5
C_{max} (µg/mL) patient 1	24	27	11.8
C_{max} (µg/mL) patient 2	17	23	35.3
$t_{1/2}$ (h) patient 1	12.3	12.7	2.7
$t_{1/2}$ (h) patient 2	6.3	7.2	15.0
4-hydroxytolbutamide			
$AUC_{0-\infty}$ patient 1 (ng/mL*h)	3112	3249	4.4
$AUC_{0-\infty}$ patient 2 (ng/mL*h)	5252	5832	11.0
C_{max} (ng/mL) patient 1	160	192	20.5
C_{max} (ng/mL) patient 2	317	431	35.8
$t_{1/2}$ (h) patient 1	15.2	13.1	-13.8
$t_{1/2}$ (h) patient 2	8.1	8.7	7.1

$AUC_{0-\infty}$ = area under the plasma concentration-time curve extrapolated to infinity; C_{max} = maximum plasma concentration; $t_{1/2}$ = elimination half-life.

Adverse events

Both evaluable patients suffered from docetaxel-related toxicities. The most severe adverse event in patient 1 was grade 3-4 neutropenia which was experienced in both study cycles. Further, in the cycle without milk thistle this patient started to develop alopecia (grade 1) and he suffered from fatigue (grade 3). In the docetaxel cycle after milk thistle supplementation this patient had fever (grade 1).

Docetaxel-related adverse events in patient 2 were fatigue (grade 3) in the cycle with milk thistle and sore throat in the non-supplemented cycle.

As shown by blood glucose checks after tolbutamide administration, no hypoglycemic events occurred in both patients.

Adherence to milk thistle intake

Reported times of milk thistle intake did not indicate non-compliance by the patients.

DISCUSSION

This study investigated the effect of a highly bioavailable milk thistle product on the pharmacokinetics of docetaxel and tolbutamide. Based on *in vitro* studies milk thistle was expected to inhibit CYP3A4 and CYP2C9, thereby increasing the systemic exposure to docetaxel and tolbutamide. Preliminary pharmacokinetic data of docetaxel, tolbutamide and 4-hydroxytolbutamide in two patients, however, did not indicate inhibition of both CYP2C9 and CYP3A4.

The pharmacokinetic results of docetaxel (Figure 2, Table 2) did not show substantial differences before and after supplementation of milk thistle. The decreased docetaxel plasma levels in patient 1 after milk thistle intake (Figure 1A) can be explained by a lower dose of 100 mg docetaxel in this cycle. This 25% dose reduction lead to a proportional decrease in $AUC_{0-\infty}$ and C_{max}. Furthermore, no major differences were observed for $t_{1/2}$, which indicates no apparent changes in docetaxel clearance and metabolism. Also in patient 2 docetaxel metabolism did not seem to be affected by milk thistle supplementation, as reflected by overlapping plasma concentration-time curves (Figure 2B). The lack of an effect on docetaxel metabolism in these patients is also reflected by the incidence of docetaxel-related adverse events, which was comparable across the two treatment cycles. Sequence effects were not likely to affect the pharmacokinetic results in this interim analysis, since both evaluable patients were randomized to two different treatment arms.

In accordance to the results of docetaxel also the CYP2C9-mediated metabolism of tolbutamide was not clearly affected by milk thistle. In theory, inhibition of CYP2C9 would have led to an increased systemic exposure to tolbutamide as indicated by an increase of $AUC_{0-\infty}$, C_{max} and $t_{1/2}$. For the CYP2C9-mediated metabolite 4-hydroxytolbutamide, these pharmacokinetic parameters should then have changed in the opposite direction. However, in this interim analysis these alterations were not clearly observed in the plasma concentration time-curves (Figure 3 and 4) and the pharmacokinetic parameters of tolbutamide and 4-hydroxytolbutamide (Table 3).

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Since the presented data are preliminary and statistical analysis was not performed, the significance of the presented pharmacokinetic results could not be determined. These preliminary data only indicate that CYP2C9 and CYP3A4 function is presumably not substantially affected by supplementation of a highly bioavailable milk thistle formulation at the recommended dose. In addition, results of CYP2C9 genotyping should also be awaited in order to clarify the obtained results. For example, the evaluated patients could have been CYP2C9 poor metabolizers and therefore less susceptible to CYP2C9 inhibition.

The pharmacokinetic profiles of docetaxel and tolbutamide were in accordance to data published in literature [17-20], thus indicating the suitability of the applied study design and bioanalytical methods to accurately execute the pharmacokinetic analyses for the current study. However, it is expected that, using the current methods, the aims of the current study can only be addressed after evaluation of the last patient.

In conclusion, preliminary results do not indicate significant pharmacokinetic interactions between milk thistle and docetaxel, and milk thistle and tolbutamide. However, definitive conclusions about the statistical and clinical relevance of the obtained results will follow

after analysis of all required patients.

ACKNOWLEDGEMENTS

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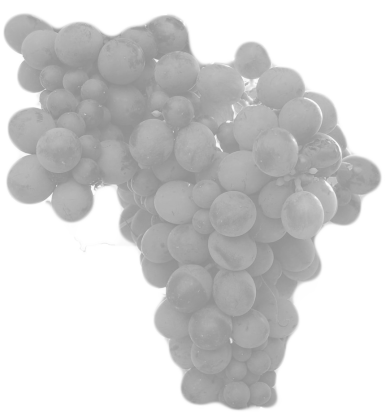
CHAPTER 6

The effect of grape seed extract on the pharmacokinetics of dextromethorphan in healthy volunteers: CYP2D6 inhibition



CHAPTER 6.1

The effect of grape seed extract on the pharmacokinetics of dextromethorphan in healthy volunteers

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Submitted for publication

ABSTRACT

Background: Grape seed extract (GSE) has been shown to inhibit the cytochrome P450 (CYP) 2D6 isoenzyme *in vitro*. For breast cancer patients using the CYP2D6 substrate tamoxifen, CYP2D6 inhibition could result in undertreatment. To determine the clinical effect of GSE on CYP2D6, the pharmacokinetic interaction between GSE and the sensitive CYP2D6 probe dextromethorphan in healthy adult volunteers was examined.

Subjects and Methods: In this open label, randomized, crossover study, thirty subjects were assigned to cohort A or B. Both cohorts ingested 30 mg dextromethorphan hydrobromide on day 1 and 10. Cohort A received 100 mg GSE capsules three times daily on day 8, 9 and 10, while cohort B started with GSE on day -1 until day 1. After urine collection (0-8 h) on day 1 and 10, the urinary dextromethorphan to dextrorphan metabolic ratio was determined.

Results: Among 28 evaluable subjects an increase of the urinary metabolic ratio was observed in 16 subjects (57%). The mean metabolic ratio (± standard deviation) increased from 0.41 (± 0.56) to 0.48 (± 0.59). This increase, however, was neither statistically (P = 0.342) nor clinically (geometric mean ratio 1.10, 90% CI (0.93-1.30)) significant. Further, the majority (73%) of the included subjects did not experience any adverse events after intake of dextromethorphan or GSE.

Conclusions: Supplementation of GSE did not significantly affect the urinary dextromethorphan to dextrorphan metabolic ratio in healthy volunteers. The results of this clinical study indicate that GSE appears to be safe to combine with drugs extensively metabolized by CYP2D6, such as dextromethorphan and tamoxifen.

INTRODUCTION

Grape seed extract (GSE, *Vitis vinifera*) is one of the top-selling herbal supplements in the United States [1]. GSE is prepared from the seeds of grapes and mainly contains monomeric catechins and proanthocyanidins, which are oligomeric catechins comprising dimers, trimers, tetramers, pentamers and their gallates [2, 3]. Examples of catechins present in GSE are epigallocatechin, catechin, epicatechin, and epicatechin gallate [3].

Proanthocyanidins have antioxidant properties and are able to increase tonicity and resistance of capillary walls [3]. Due to these properties GSE is supposed to have beneficial effects on the cardiovascular system and it is therefore used for treatment of atherosclerosis, hypertension, and hypercholesterolemia [4]. GSE has also been shown to exert chemopreventive effects in several types of cancer, including breast cancer [5].

For cancer patients, however, complementary use of GSE may not necessarily be beneficial due to the risk of pharmacokinetic interactions with their conventional chemotherapy. *In vitro* data revealed inhibition of the enzyme cytochrome P450 (CYP) 2D6 by GSE [6, 7]. For example, Nishikawa *et al*. showed that GSE was the second most potent CYP2D6 inhibitor among tested herbal extracts [6]. CYP2D6 inhibition by GSE was also demonstrated in our laboratory (data not shown). *In vitro* the IC_{50} of GSE (6.95 µg/mL) was comparable with that of the potent CYP2D6 inhibitor quinidine (IC_{50} = 8.40 µg/mL).

Considering the major role of CYP2D6 in the metabolism of the antiestrogenic agent tamoxifen [8], GSE supplementation by breast cancer patients using tamoxifen could result into undertreatment. The prodrug tamoxifen is predominantly metabolized by CYP2D6 and CYP3A4 to the metabolites 4-hydroxytamoxifen and *N*-desmethyltamoxifen, respectively. Subsequently, these metabolites are converted into endoxifen (4-hydroxytamoxifen via CYP3A4 and *N*-desmethyltamoxifen via CYP2D6). It has been shown that endoxifen is 30-100 times more potent than tamoxifen. Inhibition of CYP2D6 could thus lead to decreased systemic exposure to endoxifen, possibly resulting in decreased therapeutic efficacy. Accordingly, the potent CYP2D6 inhibitor paroxetine did significantly lower endoxifen plasma levels in breast cancer patients receiving tamoxifen [9].

The effect of GSE on the pharmacokinetics of a CYP2D6 substrate has not been reported previously in humans. Since dextromethorphan is a sensitive CYP2D6 substrate and an adequate predictor of endoxifen exposure in breast cancer patients taking tamoxifen [10], dextromethorphan would be a suitable substrate to determine the clinical effect of GSE on CYP2D6. Hence, this study investigates the effect of GSE on the pharmacokinetics of dextromethorphan and its main metabolite dextrorphan in healthy volunteers. Results of this study are relevant for establishing guidelines regarding concomitant use of GSE and tamoxifen.

METHODS

Subjects

Thirty healthy volunteers (≥18 years) were included in this study. Health status was determined by medical questionnaires. The main exclusion criteria were concomitant use of moderate or strong CYP2D6 or CYP3A4 modulating drugs (e.g. paroxetine, quinidine, erythromycin, rifampicin), CYP2D6 poor metabolizer status, use of grapefruit(juice) and more than two alcohol units per day from two weeks before study start until day 10.

Study design

The present study used an open label, randomized, cross-over design and was conducted at the David de Wied Building of the Department of Pharmaceutical Sciences of Utrecht University (Utrecht, The Netherlands).

During the screening visit, subjects had to fill out a medical questionnaire and length and height were measured. Eligible subjects were then randomized to cohort A or B. Cohort A started with intake of 15 mL of 2 mg/mL dextromethorphan hydrobromide syrup (Bisoltussin®, lot 018228, Boehringer Ingelheim bv, Alkmaar, The Netherlands) on day 1, after which urine was collected for eight hours. In the morning of day 8 each subject was reminded by a text message to start with the oral administration of GSE (Figure 1A). GSE capsules (O.P.C.-100™, 100 mg grape seed phytosome, lot 304660, Thorne Research Inc., Dover, USA) were labeled to contain: 80% (-)-epicatechin gallate, dimers, trimers, tetramers and their gallates, 15% (+)-catechin and (-)-epicatechin, and 5% pentamers, hexamers, heptamers, and their gallates [3]. These compounds were bound to phosphatidylcholine in order to enhance bioavailability. In total eight GSE capsules were administered: three times daily one capsule on day 8 and 9, and two GSE capsules in the morning and afternoon of day 10. To check the compliance of GSE intake, the subjects needed to keep diaries in which they noted the times of administration. On day 10 the subjects returned to the research facility and received the second administration of 15 mL dextromethorphan. Again, urine was collected from 0 - 8 hours after dextromethorphan intake. Cohort B started with supplementation of GSE on day -1 until day 1 (Figure 1B). The remaining study procedures on day 1 and 10 were similar as for cohort A.

For both cohorts, study ended on day 40 by assessment of adverse events via telephone calls or e-mail. Adverse events, which were also assessed on day 1 and 10, were registered according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 4.02.

The study (EudraCT number 2012-001274-28) was approved by Medical Ethical Committee of the Slotervaart Hospital (Amsterdam, The Netherlands) and was performed compliant with current standards of ICH GCP, the WHO Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO).

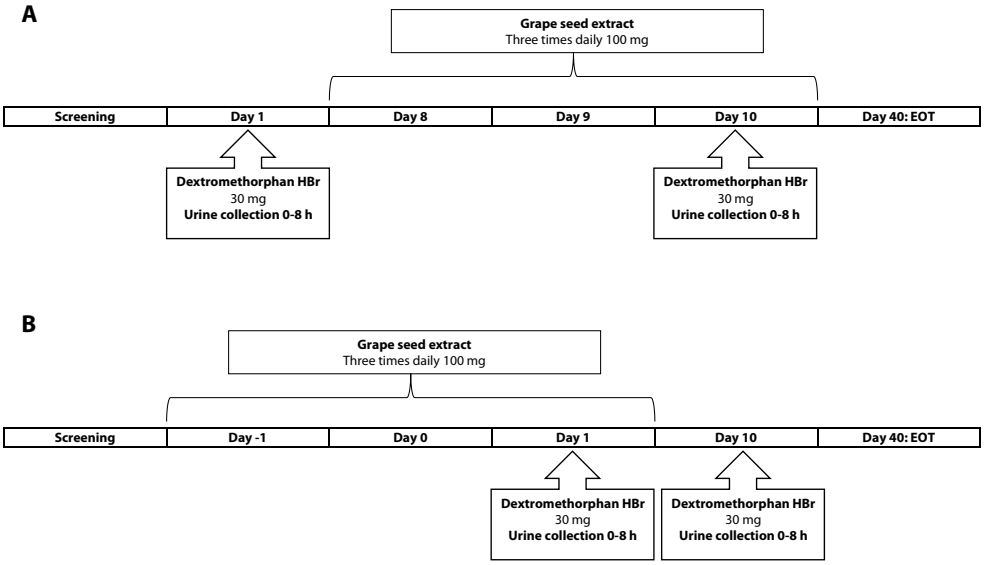

Figure 1. (A) Study design cohort A. (B) Study design cohort B.
Dextromethorphan HBr = Dextromethorphan hydrobromide; EOT = End of treatment report.

CYP2D6 genotyping

For CYP2D6 genotyping, approximately 4 mL whole blood from each included subject was collected in an EDTA tube. After inclusion of the last subjewwct, CYP2D6 genotyping was performed at the laboratory of the Division Genome Diagnostics of the Department Genetics, Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). The following CYP2D6 variant alleles were determined: *3, *4, *5, *6 and 2XN. Screening of variant alleles *3, *4, *5 and *6 would reveal the vast majority of CYP2D6 poor metabolizers in the Dutch population [11]. As CYP2D6 poor metabolizers were excluded from the final pharmacokinetic analysis, thirty subjects were included to anticipate on the exclusion of approximately 10% poor metabolizers [11].

Urinary metabolic ratio of dextromethorphan and dextrorphan

The pharmacokinetic endpoint in this study was the urinary ratio of dextromethorphan to dextrorphan, which is the most commonly used method for dextromethorphan phenotyping and has been well validated for assessment of CYP2D6 activity [12].

The concentrations of dextromethorphan and dextrorphan were determined in the collected urine on day 1 and 10 using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This LC-MS/MS assay has been validated over a concentration range of 1 – 1,000 ng/mL according to the FDA Guidelines on Bioanalytical Method Validation [13]. Validated parameters such as between-day inaccuracy (± 11.4%), within-

day precision (CV ≤ 5.55%) and between-day precision (CV ≤ 6.16%) were acceptable for both dextromethorphan and dextrorphan. Also no interference of endogenous urinary components or dextromethorphan was observed. Samples were prepared by adding 10 µL of the internal standards dextromethorphan-d3 and dextrorphan-d3 (both 10,000 ng/mL in water, Toronto Research Chemicals Inc., Toronto, ON, Canada) and 1 µL formic acid to 200 µL urine. After vortex-mixing and centrifugation for 10 min at 13,500 rpm (4°C), 150 µL of the supernatant was pipetted into an autosampler vial and finally 10 µL sample was injected into the LC-MS/MS system.

The LC-MS/MS equipment consisted of an Accela pump and autosampler and a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with heated electrospray ionization (HESI) which operated in the positive ion mode. Xcalibur software (version 2.0.7, Thermo Fisher Scientific) was used for data acquisition and processing. Injections were made on an Acquity UPLC® BEH C18 column (30 mm × 2.1 mm, d_p = 1.7 µm, Waters, Milford, MA, USA) with a VanGuard pre-column (5 mm × 2.1 mm, d_p = 1.7 µm, Waters). Liquid chromatography was carried out using 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) delivered at a flow of 0.5 mL/min. During the first minute of the run the proportion of eluent B increased from 15 to 50%. Thereafter, eluent B was kept constant at 50% for 0.7 min. Subsequently, eluent B rapidly increased to 100% for 0.8 min. Finally, the column was reconditioned at the starting conditions for 1.0 min. For dextromethorphan, internal standard dextromethorphan-d3, dextrorphan and internal standard dextrorphan-d3 the mass transitions from *m/z* 272 to 215 (collision energy -23 V), 275 to 215 (collision energy -23 V), 258 to 157 (collision energy -37 V) and 261 to 157 (collision energy -37 V) were monitored, respectively.

Concentrations of dextromethorphan and dextrorphan were obtained by calculating the ratio of the areas of the analyte and internal standard. Subsequently, the metabolic ratio (MR) of dextromethorphan/dextrorphan on day 1 was compared with MR on day 10.

Statistical analysis

To assess whether observed PK differences between cycle 1 and 2 were clinically relevant, the 90% confidence interval (CI) around the geometric mean ratio was calculated for the urinary dextromethorphan/dextrorphan ratio by use of *R* software v.2.14.0 (*R* Development Core Team, Vienna, Austria). Differences in dextromethorphan/dextrorphan ratio between cycle 1 and 2 were considered as clinically insignificant if the 90% CI of the geometric mean ratio would fall completely within the no-effect limits of 0.70 - 1.43. In addition, a paired t-test was performed on the log-transformed MR values at the 5% level for calculation of statistical significance.

Assuming an intra-subject standard deviation of 40% (range: 37 - 56%) for the urinary dextromethorphan/dextrorphan ratio [14], a sample size of at least 22 evaluable subjects was required to provide at least 80% power to demonstrate that the 90% CI of the geometric

mean ratio of the urinary ratio would fall within the no-effect range of 0.70 to 1.43 ('Power TOST' package for *R*).

RESULTS

Subjects

Between June and August 2012 thirty healthy subjects participated in the present study and completed the study according to protocol. Subject characteristics are presented in Table 1. CYP2D6 genotyping revealed that two subjects were poor metabolizers. These subjects were excluded from the pharmacokinetic analysis, which resulted in 28 evaluable subjects (18 males, 10 females). These 28 subjects were equally randomized to cohort A and B.

Table 1. Baseline subject characteristics (*n* = 30)

Gender, *n* (%)		
	Female	11 (36.7)
	Male	19 (63.3)
Age (years)		
	Median	27
	Range	20-50
Height (cm)		
	Median	178
	Range	161-191
Weight (kg)		
	Median	69.9
	Range	55.0-94.9
Race, *n* (%)		
	Caucasian	19 (63.3)
	Hispanic/Latin American	3 (10.0)
	Asian	8 (26.7)

Urinary metabolic ratio of dextromethorphan and dextrorphan

In the vast majority of the evaluable subjects the urinary dextromethorphan and dextrorphan concentrations were within the validated concentration range of 1 - 1,000 ng/mL. The samples in which concentrations of dextromethorphan and dextrorphan exceeded the upper limit of quantification were diluted with blank human urine. The calculated metabolic ratio before and after supplementation of GSE is depicted in Figure 2. It was hypothesized that inhibition of CYP2D6 by GSE would lead to an increase of the MR after GSE supplementation. Indeed, in the majority (16/28 subjects, 57%) of the subjects the MR increased after intake of GSE. Overall, the mean MR (± standard deviation) increased from 0.41 (± 0.56) to 0.48 (± 0.59). However, this increase was neither statistically ($P = 0.342$) nor clinically (geometric mean ratio 1.10, 90% CI (0.93 - 1.30)) significant.

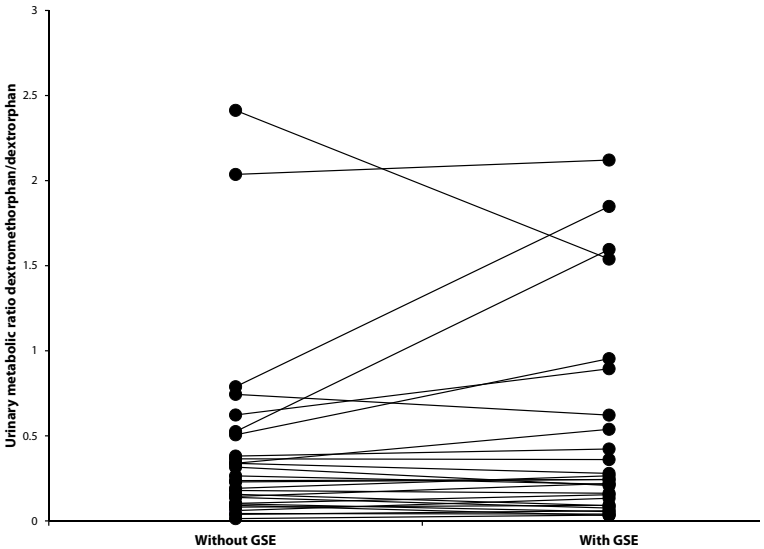

Figure 2. Individual urinary metabolic ratio (dextromethorphan/dextrorphan) before and after GSE supplementation ($n = 28$).

Adverse events

Adverse events after intake of dextromethorphan or GSE were reported in 29% of the included subjects. Dizziness (30%), headache (20%) and sleepiness (20%) were the most common adverse events.

DISCUSSION

In this study we have investigated the effect of GSE on the pharmacokinetics of dextromethorphan and its main metabolite dextrorphan. Supplementation of GSE for three consecutive days did not result in significant changes of the urinary MR of dextromethorphan and dextrorphan in healthy volunteers. Based on the observed inhibition of CYP2D6 in previous *in vitro* studies [6, 7] and in our CYP2D6 inhibition experiment (data not shown), this result was unexpected.

The lack of a significant pharmacokinetic interaction could be caused by non-compliance with the GSE intake regimen. However, inspection of the diaries and returned capsule containers did not reveal any signs of a lack of adherence.

Further, considering the direct onset of CYP2D6 inhibition, the GSE supplementation period of three days should have been sufficient to inhibit CYP2D6. For example, in the study of Samer *et al*. a single administration of the CYP2D6 inhibitor quinidine resulted in a significant increase of the urinary dextromethorphan/dextrorphan MR (0 - 8 h postdose)[15]. One could question the validity of the urine MR calculated over 8 h as an outcome measure

for CYP2D6 activity. Literature data, however, show that the urinary MR over 8 h is the most often used CYP2D6 phenotyping method [12] and has been shown to correlate significantly with the plasma $AUC_{0-\infty}$ [16]. Furthermore, the potent CYP2D6 inhibitors quinidine [15], paroxetine [17] and fluoxetine [17] significantly increased the urinary dextromethorphan/ dextrorphan ratio, which confirms the applicability of this pharmacokinetic endpoint to assess inhibition of CYP2D6.

Possibly, the bioavailability of GSE was insufficient to achieve significant inhibition of CYP2D6. It is likely that the monomeric catechins mainly contribute to the inhibitory effects of GSE on CYP2D6. First, significant inhibition of CYP2D6 *in vitro* was observed after incubating human liver microsomes with GSE normalized to 10 µM catechin [7], while proanthocyanidins such as procyanidin B2 inhibited CYP2D6 weakly (IC_{50} > 800 µM) [18]. Second, due to their smaller size monomeric catechins are better bioavailable than oligomeric proanthocyanidins. This has been demonstrated in a clinical study in which monomeric catechins reached approximately 100-fold higher plasma concentrations than a proanthocyanidin dimer [19]. Unfortunately, the bioavailability of catechin after GSE administration has not been reported in humans. However, the proanthocyanidin dimer B1 has reached a mean plasma concentration of 0.011 µM in four human volunteers two hours after a single administration of 2 g non-complexed GSE [20]. In the current study the supplementation period of GSE was longer and GSE was complexed with phosphatidylcholine, which could result in higher systemic levels of catechins and proanthocyanidins. In accordance, the bioavailabilities of ginkgo biloba [21], green tea [22] and milk thistle preparations [23] were substantially enhanced by complexing with phosphatidylcholine. Besides the use of a more bioavailable GSE formulation, it was also possible that administration of the extract in the current study would result in more potent CYP2D6 inhibition than was observed for the individual GSE components *in vitro*. Herbal components may also exert additive or synergistic effects, which has been shown for CYP3A4 inhibition by grapefruit juice [24]. Final analysis of the results, however, did not reveal similar effects for GSE. The absence of an effect on the metabolic ratio of dextromethorphan to dextrorphan in the current study indicates that, despite the use of a formulation for enhanced absorption, levels of catechins and proanthocyanidins were insufficient to affect CYP2D6.

In conclusion, the present article describes the first clinical study in which the effect of GSE on CYP2D6 was investigated. In this study GSE did not significantly alter the urinary dextromethorphan to dextrorphan metabolic ratio, which indicates that GSE can be combined safely with drugs extensively metabolized by CYP2D6, such as dextromethorphan and tamoxifen.

ACKNOWLEDGEMENTS

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6

CHAPTER 7

Conclusions and perspectives

The aim of this thesis was to explore pharmacokinetic interactions between herbal supplements and anticancer drugs in cancer patients. To address this aim, a selection of widely used herbal supplements were screened *in vitro* and subsequently tested in clinical interaction studies. The following pharmacokinetic herb-drug interactions were investigated: *Echinacea purpurea* - docetaxel (**Chapter 3.3**), St. John's wort - docetaxel (**Chapter 4.1**), milk thistle - docetaxel, milk thistle - tolbutamide (both **Chapter 5**) and grape seed extract - dextromethorphan (**Chapter 6**).

When possible, anticancer drugs were selected in this thesis as drug substrates for a specific CYP enzyme, e.g. docetaxel for CYP3A4. However, if the clinical effect of a herbal supplement on a certain CYP enzyme had not been investigated previously, then sensitive non-anticancer drugs were selected as CYP substrates [1]. Therefore, the probe drugs tolbutamide (CYP2C9) and dextromethorphan (CYP2D6) were administered in the studies with milk thistle and grape seed extract, respectively.

Based on *in vitro* results and literature data *Echinacea purpurea* and St. John's wort were expected to induce CYP3A4, possibly leading to a decreased systemic exposure to docetaxel in clinical studies. For St. John's wort this was indeed the case: the area under the concentration-time curve (AUC) of docetaxel was significantly decreased by 12%. *Echinacea purpurea*, however, did not significantly affect docetaxel pharmacokinetics.

The two other clinical studies focused on inhibition of CYP enzymes, since *in vitro* data showed that milk thistle inhibits CYP2C9 and CYP3A4, while inhibition of CYP2D6 was observed for grape seed extract. However, despite the significant inhibition of CYP enzymes by grape seed extract *in vitro*, this herbal supplement did not significantly alter the pharmacokinetics of dextromethorphan in healthy volunteers. The clinical effect of milk thistle on CYP2C9 and CYP3A4 activity in cancer patients is yet under investigation, as this clinical trial is currently ongoing. Preliminary results indicate that milk thistle does not greatly affect the pharmacokinetics of docetaxel and tolbutamide.

Overall, the clinical results described in this thesis indicate that St. John's wort should not be combined with docetaxel or other anticancer drugs extensively metabolized by CYP3A4. On the other hand, applied commercial formulations of *Echinacea purpurea* and grape seed extract at the indicated doses tested may be combined safely with substrates for CYP3A4 and CYP2D6, respectively. Based on *in vitro* studies and other available clinical data, the lack of pharmacokinetic interactions with these herbal supplements was not expected. Obviously, there is a discrepancy between results obtained *in vitro* and in humans. This finding is in agreement with the main conclusion of our review article (**Chapter 2**), which addressed the relevance of *in vitro* data for predicting herb-drug

interactions in cancer patients. In this review article it has been concluded that *in vitro* effects of dietary supplements on CYP3A4 are often not extrapolatable to clinical studies with CYP3A4 substrates.

Several factors complicate the extrapolation of *in vitro* data to clinical practice. First, the phytochemical components of herbal medicines are often poorly bioavailable. Phytochemical concentrations which significantly affect CYP enzymes *in vitro* are generally not reached in the systemic circulation due to factors such as poor solubility and degradation in the gastric environment. Most likely the low systemic exposure of the CYP3A4-modulating alkylamides in *Echinacea purpurea* contributed to the lack of a significant pharmacokinetic interaction with docetaxel (**Chapter 3.3**). In this study the major alkylamides were hardly quantifiable in plasma samples during the supplementation period and were eliminated within two hours after intake of the herbal extract. Poor bioavailability of phytochemical components, however, does not necessarily exclude the risk of pharmacokinetic interactions. For example, CYP3A4 is not exclusively located in the liver, but also in the intestinal wall. Consequently, the absorption of orally administered CYP3A4 substrates (predominantly involving intestinal CYP3A4) may be affected due to a local effect on intestinal CYP3A4 by phytochemical components that do not penetrate through the intestinal wall. This has been demonstrated for the furanocoumarins bergamottin and 6',7'-dihydroxybergamottin in grapefruit juice, which are poorly bioavailable but exhibit potent inhibition of intestinal CYP3A4 [2]. For future interaction studies, it would therefore be interesting to use the oral formulation of docetaxel, which is currently under development, as an oncolytic substrate for CYP3A4. The severity of an interaction with CYP3A4 modulating compounds will probably be greater using oral docetaxel than IV docetaxel, as both intestinal and hepatic CYP3A4 may be affected after oral administration of docetaxel. Accordingly, as reviewed in **Chapter 2** CYP3A4 induction by St. John's wort had a greater impact on the pharmacokinetics of oral midazolam compared to intravenous midazolam. Different effects on hepatic and intestinal CYP3A4 have also been shown for *Echinacea purpurea*, which significantly reduced the systemic exposure to intravenous midazolam, while the pharmacokinetics of oral midazolam were unaffected [3]. Thus, the potential of a herbal supplement to cause pharmacokinetic interactions can be different for oral and intravenous drugs. Consequently, depending on the route of administration of their anticancer drug, cancer patients may be advised differently concerning the use of a herbal supplement.

As stated in the previous paragraph, the bioavailability of *Echinacea purpurea* constituents was probably insufficient to significantly affect the pharmacokinetics of docetaxel (**Chapter 3.3**). Also milk thistle and grape seed extract are poorly bioavailable according to preclinical and clinical data. To enhance bioavailability of these herbal supplements

in the clinical studies, formulations of milk thistle and grape seed extract were used that are complexed with phosphatidylcholine. However, despite the use of this formulation grape seed extract did not significantly alter the pharmacokinetics of dextromethorphan (**Chapter 6.1**). According to preliminary results also milk thistle is not likely to significantly affect the pharmacokinetics of docetaxel and tolbutamide (**Chapter 5.1**). However, as only two of the ten required patients were evaluated, definitive conclusions of this interaction study will follow. Apparently, poor bioavailability of the tested herbal supplements remains a difficult hurdle to overcome and complicates the *in vitro – in vivo* extrapolation of pharmacokinetic interactions. In order to estimate the potential of a herbal supplement causing clinically pharmacokinetic interactions, the probability of reaching systemically relevant phytochemical concentrations should be carefully determined.

Another complicating factor of *in vitro – in vivo* extrapolation of herb-drug interactions is the intra- and interproduct variation in phytochemical content. Herbal supplements are often complex mixtures of phytochemical compounds and are, in contrast to conventional medicines, generally not standardized to a single active constituent. Standardization of herbal products is complicated when the active constituents are unknown, or when no active or analytical marker is quantified. Herbal products for which this information is lacking should be defined by the quantity of the genuine herbal substance and, if applicable, the name and composition of the extraction solvent [4]. In the absence of standardization, the ability of a herbal product to affect CYP enzymes may vary among several commercial products. For example, the extent of CYP3A4 induction by St. John's wort products depends on their hyperforin content [5]. Thus, there may be differences between the phytochemical content of extracts tested *in vitro* and in clinical studies, and also between extracts used in different clinical studies. These differences could result in inconsistent effects of a herbal extract on a particular CYP enzyme. Consequently, generalization of the results of herb-drug interaction studies for all products of a herbal extract is complicated and results are therefore only applicable to the herbal formulation tested in the clinical study. Besides differences in phytochemical content between brands, also within-batch variation can occur. Therefore, the alkylamide content of the commercially available liquid extract of *Echinacea purpurea* (Echinaforce®), which was applied in the clinical interaction study with docetaxel (**Chapter 3.3**), was investigated. Subsequently, the content of this batch was compared with four other batches of Echinaforce®. This quality control analysis revealed that the majority of the identified alkylamides were present in similar levels among the tested batches (**Chapter 3.4**), implying that the result of the interaction study is applicable for Echinaforce® liquid extracts in general. The marginal batch-to-batch variation in phytochemical content in this study, however, is not always observed for other herbal supplements as has previously been shown for St. John's wort products [6].

When herbal supplements show significant effects on CYP enzymes in *in vitro* experiments, these products should then be tested in humans using sensitive probe drugs, e.g. midazolam for CYP3A4, tolbutamide for CYP2C9, dextromethorphan for CYP2D6 and caffeine for CYP1A2 [1, 4]. If these clinical studies show significant pharmacokinetic interactions, additional interaction studies with other, generally less sensitive, CYP substrates with a narrow therapeutic window (e.g. anticancer drugs) are recommended. The advantage of using anticancer drugs as substrates for CYP enzymes is that the clinical relevance of the herbal supplementation can be assessed in the actual users of CAM: cancer patients. Since docetaxel is extensively metabolized by CYP3A4, this anticancer drug was selected as an oncolytic CYP3A4 substrate in this thesis. With regard to CYP2D6-mediated interactions, tamoxifen would be a sensitive oncolytic substrate. For other CYP enzymes, the selection of sensitive oncolytic substrates is more complicated, as multiple CYP enzymes are often involved in the metabolism of anticancer drugs. Therefore, CYP-mediated metabolic pathways of anticancer drugs should be carefully assessed in order to select an appropriate oncolytic CYP substrate,

Besides the selection of a herbal supplement and a drug substrate, also the applied study design requires attention in pharmacokinetic interaction studies. Many pharmacokinetic interaction studies follow a one-sequence crossover design, in which the substrate (alone) is administered prior to the combination with the interacting compound. Accordingly, the interaction studies with docetaxel combined with *Echinacea purpurea* (**Chapter 3.3**) and St. John's wort (**Chapter 4.1**) follow such a design. However, in order to adjust for potential period effects a randomized crossover design would be more appropriate. However, randomization in the studies with *Echinacea purpurea* and St. John's wort would not have been in the interest of patients with advanced cancer. Since induction of CYP3A4 was investigated in these studies, a longer supplementation period of the herbal supplements was required than in the studies with milk thistle and grape seed extract which were focused on the more rapid process of enzyme inhibition. If patients were randomized into the group starting with *Echinacea purpurea* or St. John's wort, they would have to wait for fourteen days prior to receiving their first course of docetaxel chemotherapy. With regard to potential tumor progression, this design was not desirable. In contrast, in the interaction studies with milk thistle (**Chapter 5.1**) and grape seed extract (**Chapter 6.1**) a shorter supplementation period was sufficient, and therefore randomization for treatment sequence was possible in these studies.

Whereas this thesis has focused on CYP enzymes, also drug transporters such as P-glycoprotein (P-gp; ABCB1) and Breast Cancer Resistance Protein (BCRP; ABCG2) affect the pharmacokinetics of many anticancer drugs, such as paclitaxel and imatinib. These drug transporters could also be affected by herbal supplements. For example, the

transcription processes of CYP3A4 and P-gp are both regulated by the nuclear pregnane X receptor. Therefore, a herbal supplement (e.g. St. John's wort) which induces CYP3A4 is also likely to upregulate P-gp expression. Thus, in the significant pharmacokinetic interaction studies between St. John's wort and imatinib [7, 8], induction of both CYP3A4 and P-gp may be the underlying mechanism. Furthermore, also the herbal supplements and components ginkgo biloba [9, 10], genistein [11] and curcumin [12] have previously shown significant interactions with P-gp substrates in healthy volunteers. Whether these herbal compounds also cause significant pharmacokinetic interactions in cancer patients using oncolytic P-gp substrates needs to be confirmed in clinical studies. These examples illustrate the need for future preclinical and clinical research to investigate the effects of herbal supplements on drug transporters.

In conclusion, the results of the pharmacokinetic studies described in this thesis contribute to the knowledge of pharmacokinetic herb-drug interactions in cancer patients. Especially for physicians these results could be helpful in informing their patients which herb-drug combinations are safe and which ones are not. The presented studies suggest that the risk of pharmacokinetic herb-drug interactions is relatively low. The often applied St. John's wort caused a significant interaction with CYP3A4 metabolized docetaxel, which may be clinically relevant. However, *Echinacea purpurea* and grape seed extract did not cause significant clinical interactions. These results demonstrate that clinical studies are needed to confirm the significant results obtained *in vitro*. Due to various factors, *in vitro* effects of herbal supplements on CYP enzymes are often not extrapolatable to the clinic. Nevertheless, considering the increased use of herbal supplements among cancer patients and potential severe clinical consequences, this area of research deserves further attention and the potential of other widely used herbal supplements to cause pharmacokinetic interactions should be investigated.

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Chemical structures of investigated molecules

Summary

Nederlandse samenvatting

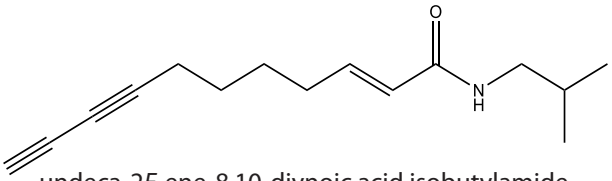
Dankwoord

List of publications

Curriculum vitae

CHEMICAL STRUCTURES OF INVESTIGATED MOLECULES

Herbal components



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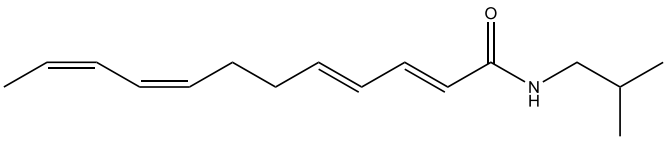
(**Chapter 3.1** and **3.4**)



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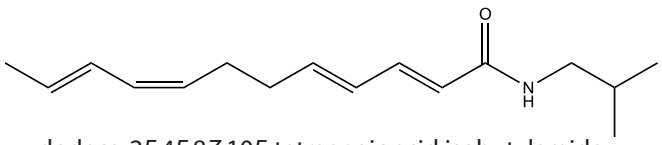
(**Chapter 3.2**, **3.3** and **3.4**)



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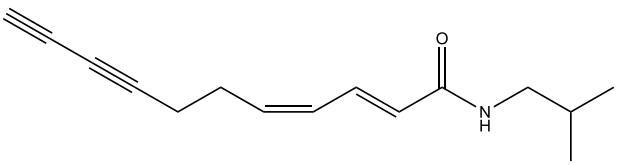

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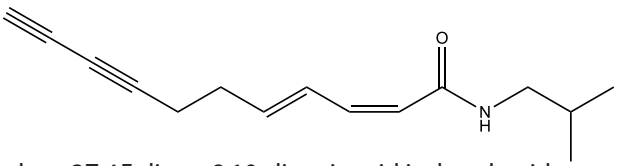
(**Chapter 3.4**)



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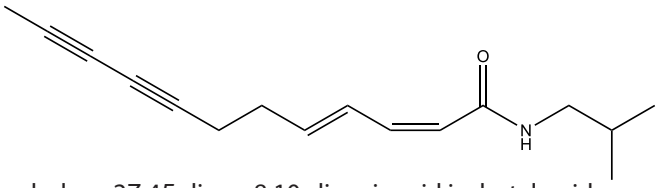

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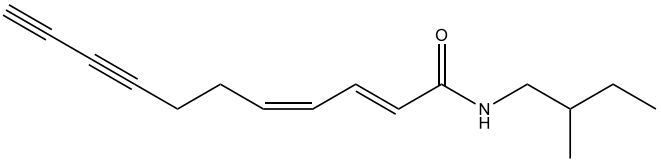
(**Chapter 3.4**)



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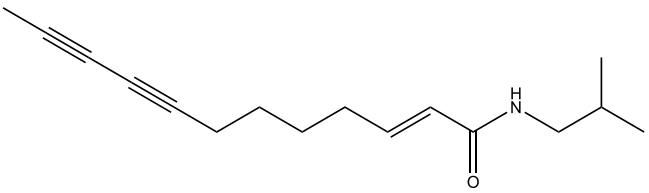

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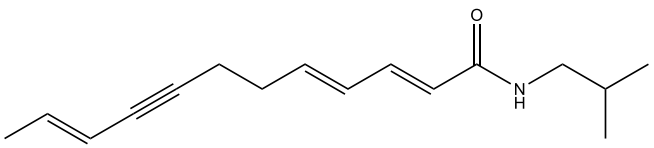
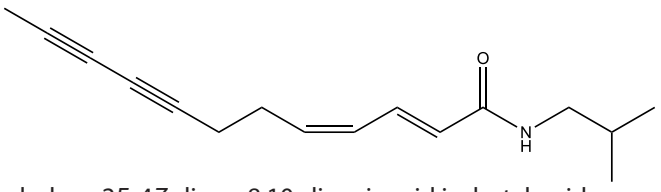
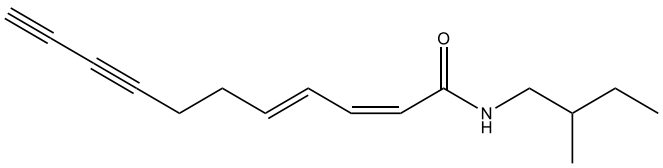
(**Chapter 3.4**)



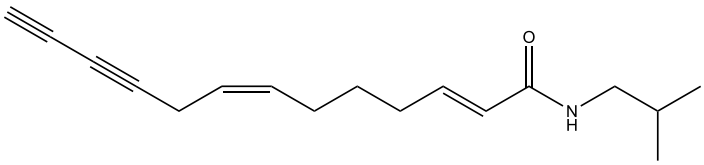
dodeca-2*E*, 4*E*, 10*E*-triene-8-ynoic acid isobutylamide
(**Chapter 3.4**)



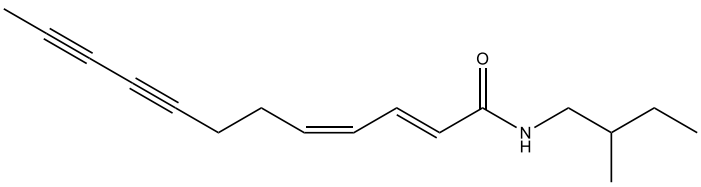
dodeca-2*E*, 4*Z*-diene-8,10-diynoic acid isobutylamide
(**Chapter 3.4**)



undeca-2*Z*, 4*E*-diene-8,10-diynoic acid 2-methylbutylamide
(**Chapter 3.4**)



trideca-2*E*, 7*Z*-diene-10,12-diynoic acid isobutylamide
(**Chapter 3.4**)



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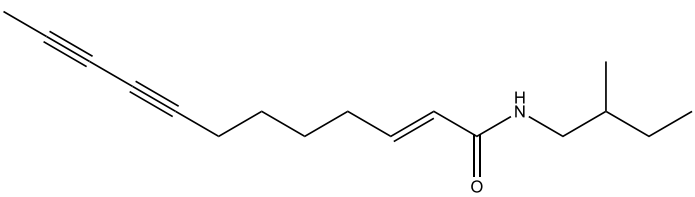
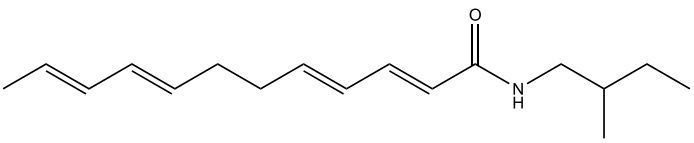
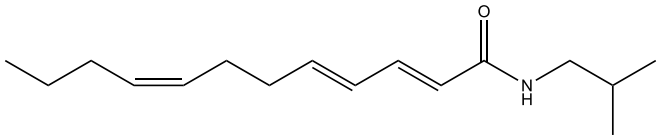
(**Chapter 3.4**)



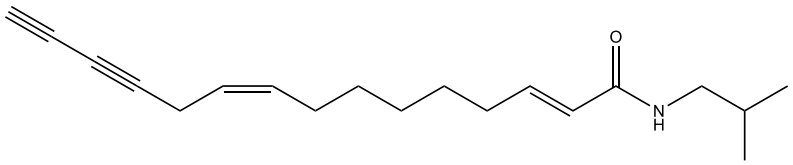
dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide
(**Chapter 3.4**)



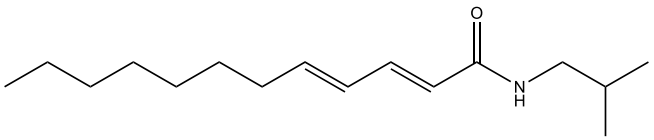
dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide
(**Chapter 3.4**)



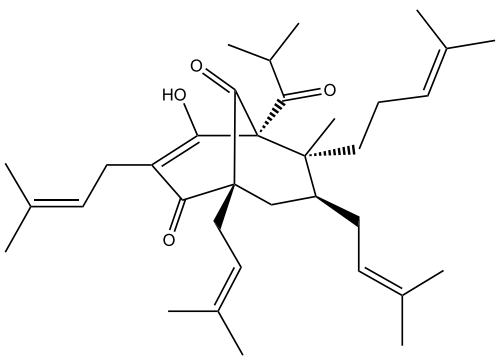
dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide
(**Chapter 3.4**)



pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide
(**Chapter 3.4**)

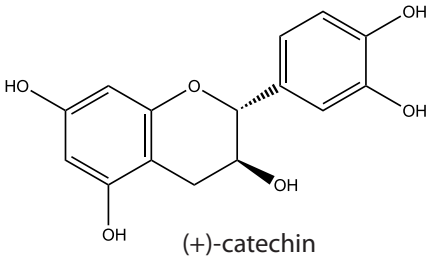


dodeca-2*E*,4*E*-dienoic acid isobutylamide
(**Chapter 3.4**)

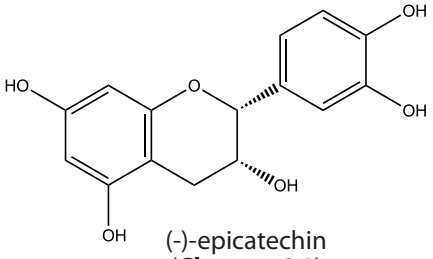


hyperforin
(**Chapter 4.1**)

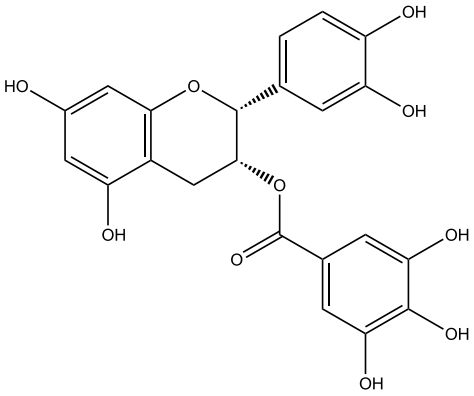
silibinin
(**Chapter 5.1**)



(+)-catechin
(**Chapter 6.1**)

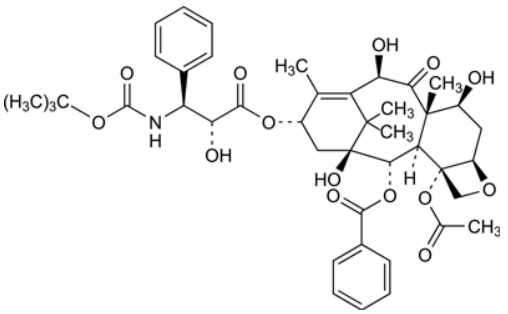


(-)-epicatechin
(**Chapter 6.1**)

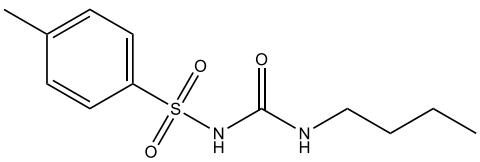


(-)-epicatechin gallate
(dimers, trimmers, tetramers, pentamers, hexamers, heptamers and their gallates not shown)
(**Chapter 6.1**)

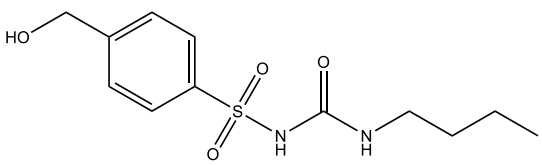
Drug substrates and metabolites



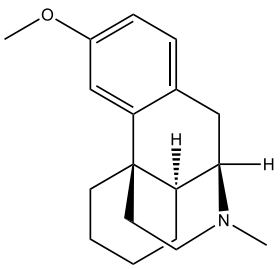
docetaxel
(**Chapter 3.3**, **4.1** and **5.1**)



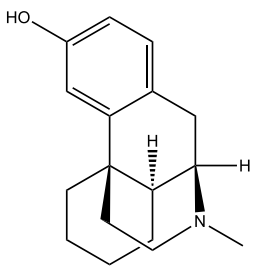
tolbutamide
(**Chapter 5.1**)



4-hydroxytolbutamide
(**Chapter 5.1**)



dextromethorphan
(**Chapter 6.1**)



dextrorphan
(**Chapter 6.1**)

SUMMARY

In cancer treatment the response to chemotherapy is often characterized by a wide interpatient variability. The use of herbal supplements by cancer patients may be one of the factors contributing to this phenomenon. Over the past decades, the popularity of herbal supplements among cancer patients has increased rapidly. Since herbal supplements have the potential to affect drug metabolizing cytochrome P450 (CYP) enzymes and drug transporters, plasma concentrations of anticancer drugs could alter due to pharmacokinetic herb-drug interactions. Consequently, patients could be undertreated or have an increased risk of toxicities, especially since the therapeutic margin of anticancer drugs is generally narrow. In order to prevent these serious clinical consequences, it is of interest to identify potential pharmacokinetic herb-drug interactions in cancer patients. However, clinical studies concerning pharmacokinetic interactions between herbal supplements and anticancer drugs are relatively scarce. Therefore, the main objective of this thesis was to investigate pharmacokinetic interactions between widely used herbal supplements (*Echinacea purpurea*, St. John's wort, milk thistle and grape seed extract) and anticancer drugs in cancer patients. Since CYP enzymes are involved in the metabolism of many anticancer drugs, the clinical studies in this thesis focused on induction and inhibition of these enzymes.

In general, prior to the execution of clinical trials the potential of herbal supplements to interact with CYP enzymes are studied *in vitro*. As reviewed in **Chapter 2.1** results obtained *in vitro* are often not extrapolatable to clinical studies. These differences are caused by several factors, such as poor pharmaceutical availability, solubility and bioavailability of phytochemical constituents. In addition, the phytochemical content of herbal supplements is often also characterized by large intra- and interproduct variation.

Chapter 2.2 replies to an article of Yaal-Hahoshen *et al*. (The Oncologist 2011;16:1197-1202) regarding the effects of the herbal mixture LCS101 on conventional chemotherapy. Yaal-Hahoshen *et al*. concluded that LCS101 prevented hematological complications in breast cancer patients receiving anthracycline- and taxane-based chemotherapy. Furthermore, addition of LCS101 to conventional chemotherapy regimens was considered both safe and feasible in patients with early breast cancer. However, these conclusions seem to be premature since the study lacks information on the hematoprotective mechanism of action of LCS101, potential pharmacokinetic interactions between LCS101 and anticancer drugs, and effects of LCS101 on the tumor.

Chapter 3 focuses on the CYP3A4 inducing effects of the popular herbal immunostimulant *Echinacea purpurea* (*E. purpurea*). Alkylamides are the major bioavailable constituents of

E. purpurea and have been shown to induce CYP3A4 in *in vitro* studies. In this chapter two bioanalytical assays based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) are presented for the alkylamides undeca-2-ene-8,10-diynoic acid isobutylamide (**Chapter 3.1**) and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (**Chapter 3.2**). These assays were developed and validated in support of the clinical interaction study with *E. purpurea* in cancer patients (**Chapter 3.3**). This clinical study showed that the applied *E. purpurea* formulation (Echinaforce®) at the recommended dose did not significantly alter the pharmacokinetics of docetaxel, which is extensively metabolized by CYP3A4. Thus, concomitant use of the tested *E. purpurea* product and docetaxel can be considered safe. Most likely, this result also applies to other batches of Echinaforce®, since the interbatch variation in alkylamide content was shown to be relatively small in a quality control analysis of multiple Echinaforce® liquid extracts (**Chapter 3.4**).

Chapter 4 focuses on the CYP3A4 inducing properties of the herbal antidepressant St. John's wort. In **Chapter 4.1** the effect of St. John's wort on the pharmacokinetics of docetaxel was investigated in patients with advanced cancer. In accordance with CYP3A4 induction, plasma levels of docetaxel were significantly decreased after supplementation with St. John's wort (Hyperiplant®, dosed as three times daily one tablet). Based on this result cancer patients are advised to refrain from Hyperiplant® tablets during chemotherapy with docetaxel in order to prevent potential undertreatment.

Besides induction of CYP enzymes, herbal supplements also have the potential to cause enzyme inhibition. For example, milk thistle, used for its hepatoprotective and detoxifying effects, has been shown to inhibit CYP2C9 and CYP3A4 *in vitro*. To investigate the clinical relevance of these findings, a clinical study was performed (**Chapter 5.1**). In cancer patients a highly bioavailable milk thistle formulation was tested in combination with tolbutamide and docetaxel, which served as substrates of CYP2C9 and CYP3A4, respectively. The interim analysis of this ongoing study showed that the pharmacokinetics of tolbutamide (including its CYP2C9-mediated metabolite 4-hydroxytolbutamide) and docetaxel were not substantially affected by milk thistle supplementation. However, definitive conclusions of this interaction study cannot be drawn prior to evaluation of the last patient.

Another popular herbal supplement is grape seed extract, which has antioxidant properties and is therefore used for treatment of, among others, atherosclerosis, hypertension and hypercholesterolemia. *In vitro* data indicated that grape seed extract potently inhibits CYP2D6, which is an essential enzyme in the biotransformation of the antiestrogenic agent tamoxifen to its active metabolite endoxifen. Therefore, the effects

of a highly bioavailable grape seed product on the urinary metabolic ratio of the sensitive CYP2D6 probe drug dextromethorphan to its CYP2D6-mediated metabolite dextrorphan was investigated in healthy volunteers (**Chapter 6.1**). In this study dextromethorphan, sharing similar metabolic pathways as tamoxifen, served as a model drug for tamoxifen. In contrast to the results obtained *in vitro*, supplementation with grape seed extract did not significantly affect the urinary metabolic ratio of dextromethorphan in this clinical study. Thus, grape seed extract appears to be safe to combine with dextromethorphan and drugs extensively metabolized by CYP2D6.

In summary, St. John's wort significantly decreased the systemic exposure of docetaxel, suggesting that this herb-drug combination should be avoided to prevent suboptimal treatment of cancer patients. In contrast, no significant pharmacokinetic interactions were observed in the clinical studies with *E. purpurea* and grape seed extract. Therefore, the investigated commercial products of *E. purpurea* and grape seed extract are considered safe to combine with CYP3A4 and CYP2D6 substrates, respectively. The clinical effects of milk thistle on CYP2C9 and CYP3A4 will be determined after final analysis of the ongoing trial.

In conclusion, this thesis shows that significant effects of herbal supplements on CYP enzymes *in vitro* are often not extrapolatable to humans. Clinical studies should therefore be performed to confirm significant results obtained *in vitro*. Considering the results of the clinical studies described in this thesis, the majority of the herbal supplements seems to pose limited risk for causing pharmacokinetic interactions with anticancer drugs.

NEDERLANDSE SAMENVATTING

Wereldwijd vormt kanker één van de belangrijkste doodsoorzaken. Dikwijls zijn de effecten van een behandeling met anti-kanker geneesmiddelen lastig te voorspellen: het anti-kanker geneesmiddel dat bij de ene patiënt een gunstig effect heeft, kan bij een andere patiënt onvoldoende werkzaam zijn of juist tot meer bijwerkingen leiden. Er zijn verschillende redenen aan te voeren voor deze variatie in respons; één daarvan zou het toenemende gebruik van kruidensupplementen, zoals St. Janskruid en *Echinacea purpurea*, kunnen zijn. De afgelopen tientallen jaren is het gebruik van kruidensupplementen onder kankerpatiënten sterk toegenomen. Uit onderzoek blijkt dat soms wel 95% van de ondervraagde patiënten dergelijke supplementen gebruiken. Doorgaans worden kruidensupplementen door patiënten als veilig beschouwd en vaak zijn artsen ook niet op de hoogte van het gebruik van kruidensupplementen. Om deze redenen wordt het gebruik van kruidensupplementen door kankerpatiënten meestal niet in verband gebracht met een verminderde werking van chemotherapie of een verhoogd risico op bijwerkingen. Onderzoek in cellen en celsystemen (*in vitro* onderzoek) en klinische studies hebben echter aangetoond dat bepaalde kruidensupplementen de omzetting (metabolisme) van anti-kanker geneesmiddelen in het lichaam kunnen remmen of stimuleren. Bij deze effecten op het metabolisme (farmacokinetische interacties) zijn vaak cytochroom P450 (CYP) enzymen, zoals CYP3A4, CYP2C9 en CYP2D6, betrokken. CYP-enzymen zijn voornamelijk in de lever gelokaliseerd en spelen vaak een belangrijke rol bij het metabolisme van anti-kanker geneesmiddelen. Door remming (inhibitie) of stimulatie (inductie) van de activiteit van CYP-enzymen kunnen de bloedconcentraties van anti-kanker geneesmiddelen worden beïnvloed waardoor de werking van deze geneesmiddelen kan worden versterkt of verminderd. Om het risico op deze ernstige gevolgen te verlagen, is het van belang om potentiële farmacokinetische interacties tussen kruidensupplementen en anti-kankergeneesmiddelen in kankerpatiënten te bestuderen. Echter, tot op heden zijn er nog weinig van dergelijke studies uitgevoerd. Het doel van het onderzoek beschreven in dit proefschrift was daarom om de klinische effecten van een aantal veelgebruikte kruidensupplementen op het metabolisme van anti-kanker geneesmiddelen te onderzoeken. Deze effecten kunnen worden bepaald door het meten van de concentraties van de anti-kanker geneesmiddelen in lichaamsvloeistoffen, zoals bloedplasma of urine.

Klinische interactiestudies worden meestal voorafgegaan door *in vitro* onderzoek waarin de effecten van kruidensupplementen op CYP-enzymen worden bestudeerd.
Door middel van het vergelijken van studies uit de literatuur wordt in **Hoofdstuk 2.1** de voorspellende waarde van *in vitro* onderzoek voor klinische interactiestudies beoordeeld. Uit dit hoofdstuk blijkt dat effecten van kruidensupplementen in *in vitro* studies vaak niet

overeenkomen met effecten in de mens. Een aantal factoren die bij deze verschillen in uitkomsten een rol kunnen spelen zijn: matige afgifte uit de toedieningsvorm, matige oplosbaarheid en matige biologische beschikbaarheid van de plantaardige inhoudstoffen. Verder is gebleken dat de samenstelling van plantaardige inhoudstoffen sterk kunnen verschillen tussen verschillende merken en zelfs tussen verschillende batches van hetzelfde kruidenpreparaat.

In **Hoofdstuk 2.2** staat een reactie op een artikel van Yaal-Hahoshen *et al*. (The Oncologist 2011;16:1197-1202) over de effecten van het Chinese kruidenmengsel LCS101 op anti-kanker geneesmiddelen. De conclusie van het artikel luidde dat LCS101 bijwerkingen van het bloedbeeld kan voorkomen bij borstkankerpatiënten die met chemotherapie worden behandeld. Het gebruik van LCS101 naast reguliere anti-kanker geneesmiddelen bij borstkankerpatiënten werd als veilig en wenselijk beschouwd. Deze conclusies lijken echter prematuur, vanwege het ontbreken van informatie over het werkingsmechanisme van LCS101, mogelijke farmacokinetische interacties tussen LCS101 en anti-kanker geneesmiddelen en effecten van LCS101 op de tumor.

Hoofdstuk 3 richt zich op de inducerende effecten van het populaire kruidensupplement *Echinacea purpurea* (*E. purpurea*) op het enzym CYP3A4. Alkylamides zijn de voornaamste componenten in *E. purpurea* die biologisch beschikbaar zijn en waarvan *in vitro* CYP3A4-inductie is aangetoond. **Hoofdstukken 3.1** en **3.2** beschrijven twee bioanalytische methoden voor de kwantitatieve bepaling van alkylamides in humaan plasma op basis van vloeistofchromatografie gekoppeld met tandem massa spectrometrie (LC-MS/MS). Deze methoden werden gebruikt ter ondersteuning van een klinische studie waarin de farmacokinetische interactie tussen *E. purpurea* (Echinaforce®, A. Vogel) en het anti-kanker geneesmiddel én CYP3A4-substraat docetaxel werd onderzocht (**Hoofdstuk 3.3**). Docetaxel is een zeer geschikt geneesmiddel om interacties via CYP3A4 te bestuderen, omdat dit middel voornamelijk door CYP3A4 wordt gemetaboliseerd. In de klinische studie had inname van Echinaforce® gedurende 14 dagen volgens de geadviseerde dosering van 3 maal daags 20 druppels geen significant effect op de plasmaconcentraties van docetaxel. Gelijktijdig gebruik van Echinaforce® volgens de geadviseerde dosering en docetaxel kan daarom als veilig worden beschouwd. Verder is uit een kwaliteitscontrole van vijf verschillende Echinaforce® batches (**Hoofdstuk 3.4**) gebleken dat er weinig verschil was in de alkylamide-samenstelling van deze batches. De conclusie van de klinische studie is daarom ook van toepassing op andere batches van Echinaforce®.

Hoofdstuk 4 beschrijft de inductie van CYP3A4 door het plantaardige antidepressivum St. Janskruid. In **Hoofdstuk 4.1** wordt de farmacokinetische interactie tussen St. Janskruid en docetaxel bestudeerd. Inname van St. Janskruid (Hyperiplant®, VSM) in een dosering

van 3 maal daags 1 tablet gedurende 14 dagen bleek de concentratie van docetaxel in het bloed van kankerpatiënten significant te verlagen. Dit effect was verwacht op basis van eerder gerapporteerde CYP3A4-inductie door St. Janskruid. Om mogelijke onderbehandeling van kankerpatiënten te voorkomen, wordt gelijktijdig gebruik van Hyperiplant® en docetaxel niet geadviseerd.

Naast inductie zijn sommige kruidensupplementen ook in staat om CYP-enzymen te remmen. Zo hebben *in vitro* studies remming van CYP2C9 en CYP3A4 door het supplement mariadistel aangetoond. Mariadistel wordt veelvuldig gebruikt ter ondersteuning van de lever. De klinische relevantie van de *in vitro* resultaten wordt momenteel onderzocht in een studie met mariadistel (3 maal daags 180 mg gedurende 4 dagen) in combinatie met docetaxel (CYP3A4-substraat) en tolbutamide (CYP2C9-substraat) in kankerpatiënten (**Hoofdstuk 5.1**). Deze klinische studie is nog lopende. Voorlopige resultaten wijzen uit dat de plasmaconcentraties van docetaxel en tolbutamide niet significant veranderen na inname van mariadistel. Definitieve conclusies kunnen echter pas worden getrokken na evaluatie van de meetgegevens van de laatste patiënt.

Een ander veelgebruikt kruidensupplement is druivenpitextract. Druivenpitextract wordt gebruikt bij de behandeling van onder andere aderverkalking, hoge bloeddruk en een verhoogd cholesterolgehalte. *In vitro* resultaten tonen aan dat druivenpitextract het enzym CYP2D6 krachtig remt. Dit gegeven zou van belang kunnen zijn voor borstkankerpatiënten die tamoxifen gebruiken. CYP2D6 speelt namelijk een belangrijke rol bij de omzetting van tamoxifen naar de actieve stof endoxifen. Remming van CYP2D6 zou kunnen leiden tot een verminderde vorming van endoxifen waardoor mogelijk het gewenste therapeutische effect niet wordt bereikt. **Hoofdstuk 6.1** beschrijft het effect van druivenpitextract (3 maal daags 100 mg gedurende 3 dagen) op het metabolisme van dextromethorfan in gezonde vrijwilligers. Dextromethorfan, een hoestprikkeldempend middel, wordt op vergelijkbare wijze als tamoxifen gemetaboliseerd. In tegenstelling tot de *in vitro* resultaten had druivenpitextract geen significant effect op het metabolisme van dextromethorfan bij gezonde vrijwilligers. De conclusie van deze studie luidt dat druivenpitextract veilig te combineren is met dextromethorfan en ook met andere geneesmiddelen die voornamelijk door CYP2D6 worden gemetaboliseerd, zoals tamoxifen.

Samenvattend, St. Janskruid (Hyperiplant®) veroorzaakte een significante verlaging van de plasmaconcentraties van docetaxel. Om een verminderde werkzaamheid van docetaxel te voorkomen, is het niet raadzaam om deze middelen gelijktijdig te gebruiken.
In de klinische studies met *E. purpurea* en druivenpitextract werden geen significante farmacokinetische interacties gevonden, terwijl de effecten van mariadistel pas na afloop

van de lopende studie kunnen worden bepaald.

Concluderend, dit proefschrift laat zien dat significante *in vitro* resultaten meestal niet te extrapoleren zijn naar de klinische praktijk. Klinische studies zijn daarom nodig voor de bevestiging van significante *in vitro* resultaten. Op basis van de uitgevoerde klinische studies lijken de meeste kruidensupplementen, ingenomen volgens de standaarddosering, veilig te combineren met anti-kanker geneesmiddelen. Een uitzondering hierop vormt St Janskruid: gelijktijdig gebruik bij behandeling met chemotherapie welke wordt gemetaboliseerd via CYP3A moet worden afgeraden.

DANKWOORD

Naast het bloed (hoofdstukken 3.1 en 6.1), zweet (alle hoofdstukken) en urine (hoofdstuk 6.1) wat het onderzoek beschreven in dit proefschrift mijzelf heeft gekost, zou dit proefschrift niet tot stand zijn gekomen zonder de hulp van een groot aantal personen. Door het veelzijdige karakter van mijn onderzoek en de nauwe samenwerking tussen de instituten Universiteit Utrecht, NKI-AVL en het Slotervaartziekenhuis heb ik de afgelopen 4,5 jaar met veel mensen en afdelingen mogen samenwerken.

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Andrew
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LIST OF PUBLICATIONS

Manuscripts

Goey AKL, Mooiman K, Beijnen JH, Schellens JHM, Meijerman I – Relevance of *in vitro* and clinical data for predicting CYP3A4-mediated herb-drug interactions in cancer patients. Cancer Treat Rev. 2013. *Article in press*.

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Abstracts

Goey AKL, Meijerman I, Rosing H, Marchetti S, Mergui-Roelvink M, Keessen M, Burgers JA, Beijnen JH, Schellens JHM - Pharmacokinetic interaction between St. John's wort and docetaxel in patients with advanced cancer. Oral presentation at the Scientific Spring Meeting of the Dutch Society for Clinical Pharmacology and Biopharmacy (NVKFB), 26 March 2013, Utrecht, The Netherlands.

Goey AKL, Meijerman I, Rosing H, Keessen M, Beijnen JH, Schellens JHM – Phase I Interaction Study of Docetaxel with Supplementation of St. John's wort. Cancer Research: April 15, 2012;72(8), supplement 1. Poster presentation at the AACR Annual Meeting 2012, 31 March - 4 April 2012, Chicago (IL), USA.

Goey AKL, Meijerman I, Rosing H, Keessen M, Beijnen JH, Schellens JHM – Phase I Interaction Study of Docetaxel with Supplementation of *Echinacea purpurea*. Poster presentation at the FIGON Dutch Medicines Days 2011, 3 - 5 October 2011, Lunteren, The Netherlands.

CURRICULUM VITAE

Andrew Goey werd geboren op 14 oktober 1983 te Heerlen. In zijn jeugd is hij menigmaal verhuisd en groeide daardoor op in Landgraaf, Uithoorn, Frederick (VS), Ridderkerk en Goirle. In 1996 begon hij zijn middelbareschooltijd aan het Erasmiaans Gymnasium te Rotterdam. Halverwege de opleiding verhuisde hij in 1999 naar Goirle alwaar hij in 2002 zijn gymnasiumdiploma aan het Mill-Hillcollege behaalde.

Vervolgens begon hij in september 2002 met de bacheloropleiding Farmacie aan de Universiteit Utrecht. Na het behalen van zijn bachelordiploma in augustus 2005 begon hij in september 2005 aan de masteropleiding Farmacie aan de Universiteit Utrecht. Tijdens de masteropleiding Farmacie voerde hij van september 2006 tot maart 2007 het onderzoek 'Development and Validation of Novel Mouse Models of Bipolar Mania' uit in het laboratorium van prof.dr. M.A. Geyer van de Department of Psychiatry, University of California, San Diego (UCSD), VS. Het masterdiploma Farmacie werd in augustus 2008 behaald.

In oktober 2008 begon hij met het promotieonderzoek dat beschreven is in dit proefschrift aan de afdeling Farmaco-epidemiologie & Klinische Farmacologie aan de Universiteit Utrecht en de afdeling Klinische Farmacologie in het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis te Amsterdam. Dit onderzoek werd uitgevoerd onder begeleiding van prof.dr. J.H.M. Schellens, prof.dr. J.H. Beijnen en mw. dr.ir. I. Meijerman. Naast het promotieonderzoek werd tevens de opleiding tot klinisch farmacoloog gevolgd. Vanaf juli 2013 zal Andrew als postdoc-onderzoeker, onder leiding van dr. W.D. Figg, werkzaam zijn in het Clinical Pharmacology Program van het Center for Cancer Research, onderdeel van het National Cancer Institute te Bethesda (Maryland, VS).

Andrew Goey was born on October 14 1983 in Heerlen, The Netherlands. During his youth, Andrew has moved several times and therefore he grew up in Landgraaf, Uithoorn, Frederick (USA), Ridderkerk and Goirle. In 1996 he started secondary school at Erasmiaans Gymnasium in Rotterdam. After moving to Goirle in 1999 he completed secondary school at Mill-Hillcollege in 2002.

In September 2002 he started the Bachelor's program in Pharmacy at Utrecht University. After obtaining his Bachelor's degree in August 2005 he continued with the Master's program Pharmacy at Utrecht University. During the Master's program he executed the research project 'Development and Validation of Novel Mouse Models of Bipolar Mania' at the laboratory of prof.dr. M.A. Geyer at the Department of Psychiatry at the University of California, San Diego (UCSD), USA, from September 2006 until March 2007. He completed his Master's program in August 2008.

In October 2008 he started with the PhD project described in this thesis at the Department of Pharmacoepidemiology and Clinical Pharmacology at Utrecht University and the Department of Clinical Pharmacology at The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital in Amsterdam. This work was supervised by prof.dr. J.H.M. Schellens, prof.dr. J.H. Beijnen and mw. dr.ir. I. Meijerman. During his PhD project he also started the training in Clinical Pharmacology.

As of July 2013, Andrew will start working as a postdoctoral researcher under supervision of dr. W.D. Figg in the Clinical Pharmacology Program of the Center for Cancer Research at the National Cancer Institute in Bethesda (Maryland, USA).