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Trade Herbal Products and Induction of CYP2C19 and CYP2E1 in Cultured Human Hepatocytes

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Abstract: The aim of this study was to evaluate *in vitro* the dose-dependent induction potential of six commonly used trade herbal products on CYP2C19 and CYP2E1 metabolic activities in cultured human hepatocytes. S-mephenytoin and chlorzoxazone were used as specific CYP substrates, respectively, and rifampicin was used as a positive induction control for both enzymes. The hepatocytes were exposed to herbal extracts in increasing and biological relevant concentrations for 72 hrs and CYP substrate metabolites were quantified by validated HPLC methodologies. The major findings were that St John's wort was the most potent CYP-modulating herb, showing a dose-dependent induction/inhibition of both CYP2C19 and CYP2E1, with induction at low dosages and inhibition at higher. *Ginkgo biloba* showed an induction/inhibition profile towards CYP2C19 which was similar but weaker than that observed for St John's wort. If cooperative mechanisms are involved is still an open question. Common sage induced CYP2C19 in a log-linear dose-dependent manner with increasing concentrations. Common valerian was a weak inducer of CYP2C19, while horse chestnut and cone flower were characterized as non-inducers of CYP2C19. Only St John's wort showed an inductive effect towards CYP2E1. In addition to St John's wort, Gingko biloba and common sage should be considered as possible candidates for clinically relevant drug-herb interactions with selected CYP2C19 substrates.

In recent years, several reports have been published regarding the modulation of cytochrome P450 enzyme (CYP) activities by natural products. Sub-cellular fractions, such as liver microsomes or isolated CYP isozymes, have been used for the investigation of CYP inhibition *in vitro*. *In vitro* induction can, however, only be studied in an intact cellular system like cultured human hepatocytes. Despite the unique abilities exhibited by human hepatocytes for studying CYP induction, reports using this system in the evaluation of possible drug-herb interactions are limited. Nevertheless, CYP3A4 induction by St John's wort, common valerian, garlic and grape-seed has been reported [1,2] and also CYP3A4 and CYP1A2 induction by *Ginkgo biloba* [2,3].

CYP2C19 is a major metabolizing enzyme of several clinically important drugs such as proton-pump inhibitors like omeprazole and lanzoprazole, anti-epileptics-like mephenytoin, diazepam and selective serotonin reuptake inhibitors like citalopram [4]. This enzyme is expressed in liver but also in the small intestine [5] and can thus also be involved in presystemic metabolism of medicines.

CYP2E1 is involved in the liver bioactiviation of many environmental xenobiotics creating reactive metabolites, reactive oxygen species [6–8] and is also involved in the metabolism of drugs like chlorzoxazone and enflurane [9]. An induction of these two CYP enzymes, by a regular or non-regular intake of inducing herbs, may therefore be of importance for the efficacy of several drugs and for general toxicity.

Previous investigations in man have shown that CYP2C19 activity is susceptible to induction by herbs and natural products; examples include St John's wort, *G. biloba* and the Chinese herbal mixture Yin Zhi Huang (also called Jaundiclear) [10–12]. CYP2E1 activity has been shown to be induced by St John's wort and inhibited by garlic oil in man [13]. Except for these few herbs, only minor information is available on inductive effects of herbs on CYP2C19 and CYP2E1 metabolism.

The present study focuses on the dose-dependent inductive potential of six commonly used herbs on CYP2C19 and CYP2E1-mediated drug metabolism in cultured primary human hepatocytes. For the six herbal products investigated, St John's wort (*Hypericum perforatum*), common valerian (*Valeriana officinalis*), common sage (*Salvia officinalis*), horse chestnut (*Aesculus hippocastanum*), *G. biloba* and cone flower (*Echinacea Purpurea*), no data are previously published on their inductive effects on CYP2C19 and CYP2E1 in a human hepatocyte model. For common valerian, common sage, horse chestnut and cone flower, no data at all are available on their inductive potential towards CYP2C19 or CYP2E1.

Materials and Methods

Chemicals, reagents, and herbs. S-mephenytoin (Sigma UC-175), rifampicin (Sigma R-5777), chlorzoxazone (Sigma C-4397), 6-hydroxychlorzoxazone (Sigma UC-148, product of chlorzoxazone hydroxylation), 4-hydroxymephenytoin (Sigma H-146, product of

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Table 1.

Overview of extraction parameters for herbs

Herb	Solvent for extraction and stock [% EtOH]	Weight, one pill or capsule [mg]	Weight, dried extract [mg]	Volume for extraction [ml]	Concentration stock solution [mg/ml]
St John's wort	60	538	477	25 + 8	51.8
Common valerian	60	508	316	25 + 8	78.3
Common sage	0*	291	150	20 + 6	58.1
Horse chestnut	50	384	295	20 + 6	122
Ginkgo biloba	50	262	205	15 + 5	61.5
Cone flower	20	3.05†	96.6	–	138

*extracted in water; †liquid; 3 ml (3.05 g) was evaporated, weighed and redissolved 20% EtOH.

mephenytoin hydroxylation), collagenase (Sigma C-0130), hyaluronidase (Sigma H-3506), gentamicin (Sigma G-3632), amikacin (Sigma A-2324), albumin (Sigma A-8022), HEPES (Sigma H-3375), penicillin-streptomycin (Sigma P-0781), D-fructose (Sigma F-3510), collagen (Sigma C-7661), β-NADPH (Sigma N-1630), Dulbecco's modified eagle's medium (Sigma D-2902), and insulin (Sigma I-6634) were obtained from Sigma-Aldrich (St Louis, MO, USA). St John's wort (Hypericum Stada®, Stada Arzneimittel AG, batchno. 4523), *G. biloba* (Seredrin®, Bioplanta Arzneimittel GmbH, batch no. 3,090,103), common sage (Nosweat®, Biokraft Pharma AB, batch no. JH561), common valerian (Valerina® Forte, Pharbio Medical Int. AB, batch no. 010,411), horse chestnut (Venastat®, Boehringer Ingelheim International GmbH, batch no. 40,230,906) and cone flower (Echinagard®, Madaus AG, batch no. F0241165) were purchased at a local pharmacy in Norway.

Preparation of herbal extracts. Dry commercial herbal products (one pill or one capsule) were grounded in a mortar and dissolved in an appropriate solvent for extraction (table 1). The same extraction solvent was applied for each product as that originally used by the producer: ethanol or water. The grounded herbal product was dissolved in solvent (15–25 ml) and extracted with constant stirring at 30° for 1 hr while protected from sunlight. Solvent containing herbal constituents was centrifuged for 10 min. at approximately 1600 ×*g* and decanted off into a pre-weighed beaker. The remaining sediment was re-extracted and the procedure was repeated. The pooled solvent extract was evaporated at 40°. The herbal residue was weighed and re-dissolved in a small volume of solvent producing concentrated herbal stock solutions. All herbal stock solutions were kept at 4°, avoiding light. Shelf life was set to 2 weeks.

Hepatocyte isolation and induction. Human primary hepatocytes were prepared from donation in accordance with the Shanghai Donation Regulation (China) and informed consent rule (2001). The donor was a 28-year-old Chinese male (Research Institute for Liver Diseases donor number GFHQ-2). Trauma was the cause of death. All serology data (human immunodeficiency virus, hepatitis B virus, hepatitis C virus and syphilis) were normal. No data were available on smoking habits. The basic CYP2C19 enzyme activity in our hepatocytes was compared with data of genotyped livers previously obtained from the laboratory. On this basis, our liver was categorized as being obtained from a 'normal CYP2C19 metabolizer'. Human hepatocytes were prepared by a collagenase perfusion technique described by others [14]. Viability of prepared human primary hepatocytes was determined by the trypan blue exclusion method and hepatocytes were accepted for experimental use if viability was higher than 70%. Primary human hepatocytes were plated on Falcon 24-well culture plates (0.5 ml, 0.35×10^6 cells/well) coated with collagen in Dulbecco's modified eagle's medium, supplemented with insulin, albumin, D-fructose, gentamicin, amikacin, penicillin streptomycin, and foetal calf serum. After 2–4 hrs, the sample wells were inspected to ensure cell attachment. After 12 hrs, a monolayer of cells was formed and unattached cells were removed by gentle agitation and the medium was replaced with a serum-free medium (incubation medium) containing all the supplements previously mentioned. The incubation medium was changed every 24 hrs. Culture plates with cells were maintained at 37° in an atmosphere of 5% CO_2 and 95% air. At 24, 48, and 72 hrs, the cells were exposed to 0.5-ml incubation medium containing a positive CYP2C19 and CYP2E1 control inducer (rifampicin 50 μM, 41.2 μg/ml) or herbal extracts in increasing concentrations.

Cytochrome P450 metabolism. At 24, 48, 72, and 96 hrs, the medium was aspired and non-treated cells were exposed to incubation medium containing probe substrates of CYP2C19 (s-mephenytoin 100 μM, 21.8 μg/ml) or CYP2E1 (chlorzoxazone 500 μM, 84.8 μg/ml) for 1 hr at 37° to establish basal levels of CYP activities. Incubations were stopped by adding 0.5 ml ice cold methanol. Samples were transferred to centrifuge tubes, centrifuged at 1400 ×*g* for 5 min. and 500 μl of the supernatants were transferred to HPLC vials.

After 96 hrs, incubation medium containing probe substrates was added to the cells previously exposed to the positive control inducer or herbal extracts and incubated as described above.

HPLC conditions. Fifty-microlitres incubation samples containing 4-hydroxy-mephenytoin (product of CYP2C19-mediated metabolism of s-mephenytoin) or 6-hydroxychlorzoxazone (product of CYP2E1-mediated metabolism of chlorzoxazone) were directly injected into an HPLC system (Mobile phase: A: 0.05% phosphoric acid, pH 3, B: methanol; flow: 1 ml/min.; column: Luna C18 5 μm (150 × 4.6 mm); gradient programme: from 25% B up to 50% B in 20 min. for 4-hydroxy-mephenytoin and 40% B (for 11 min.), 45% B (for 5 min.), 40% B (for 2 min.) for 6-hydroxychlorzoxazone, using UV detection at 214 or 287 nm, respectively, for quantitation.

Calculation of CYP activity and limit of quantitation. Metabolite peak areas were used to calculate CYP activities. Eight-point standard curves: 4-hydroxy-mephenytoin (CYP2C19), range 0.065–4.23 μM, $r^2 \geq 0.996$; 6-hydroxy-chlozoxazone (CYP2E1), range 0.095–4.45 μM, $r^2 \geq 0.997$ were constructed. Enzyme activity was calculated and normalized per million cells (0.35) and an incubation time of 60 min. Enzyme activity was expressed as picomole metabolite formed $\times 10^6$ cells^{-1} $\times$ min.$^{-1}$ (picomole $\times 10^6$ cells^{-1} $\times$ min^{-1}). No internal standard was used. Limits of quantitation for 4-hydroxy-mephenytoin and 6-hydroxychlorzoxazone were 65 and 95 nM, respectively. Inter- and intra-day assay variations were less than 10% in the investigated concentration ranges.

Statistics. Data are presented as means ± S.D. of three replicates. A two-sample t-test was used to test the effect of herbal preparations or the positive control inducer on CYP enzyme activities versus controls. Statistical analysis was performed on SPSS (SPSS for Windows, Release 13.0. 2004, SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

Table 2.

Day-to-day variation for basic enzyme activities

	Enzyme activity* [picomole × 10^6 cells^{-1} × min^{-1}]			
CYP isozyme	24 hr	48 hr	72 hr	96 hr
CYP2C19	13.7 ± 0.3	14.8 ± 0.4	14.4 ± 0.2	14.3 ± 0.5
CYP2E1	18.8 ± 0.2	20.2 ± 0.3	20.6 ± 0.2	19.6 ± 0.6

*mean ± SD for 3 parallels each day.

Results

The basic control activities for CYP2C19 and CYP2E1 metabolism in the non-treated hepatocytes were 14.3 ± 0.5 and 19.6 ± 0.8 picomole × 10^6 cells^{-1} × min^{-1}, respectively (control activities for each day are given in table 2). There was a 4.4% and 4.2% difference in activities when comparing activities for first and last day. In fig. 1 is shown that CYP2C19 activity was increased by 125 ± 7% ($P < 0.05$) in the rifampicin-treated cells, while fig. 2 shows that CYP2E1 activity was increased by 33 ± 5% ($P < 0.05$).

Extracts from the six different commercial herbal products were tested at three different concentrations. These are expected to cover the biological range of concentrations obtained in man. In fig. 1 is shown that the CYP2C19 activity was increased by St John's wort, common valerian, *G. biloba* and common sage, with a maximum increase of 134%, 57%, 75%, and 100%, respectively. St John's wort increased the metabolic activity of CYP2C19 only at the two lowest concentrations tested, while a strong inhibition was observed at the highest, thus demonstrating a dose-dependent induction/inhibition effect. *Ginkgo biloba* showed a similar pattern and a linear log dose-dependent effect was found ($y = -50.1x + 195.2$, $r^2 = 0.993$, $P < 0.05$) between the decrease in CYP2C19 activity and log increase in the concentration of *G. biloba*. In Contrast to *G. biloba,* common sage increased CYP2C19 activity with increasing concentrations in a linear log dose-dependent manner ($y = 35.0x + 98.4$, $r^2 = 0.985$, $P < 0.05$). Apart from horse chestnut and cone flower, which showed no inductive potential towards CYP2C19 activity, common valerian demonstrated the lowest induction potential of CYP2C19 at the dosages tested. However, an equal increase in CYP2C19 activity of 57% was found for both the two lowest concentrations applied.

In fig. 2 is shown that St John's wort increased CYP2E1 activity by 65% ($P < 0.05$); also this at the lowest concentration tested. At higher concentrations, St John's wort showed inhibitory effects towards CYP2E1 activity without, however, any sort of a linear correlation. The other herbal preparations showed either weak inhibitory or no effect towards CYP2E1-mediated metabolism.

Discussion

Control experiments with the positive control inducer rifampicin showed that rifampicin was a far better inducer of CYP2C19 activity than of CYP2E1 activity, but nevertheless considered sufficient for confirming the CYP functionality of the hepatocyte system.

Ethanol and water extracts of trade herbal products were investigated in this study. By introducing alcohol in CYP induction studies, methodological problems could arise. Especially with CYP2E1 as this isoform is reported to be induced in rats by low ethanol concentrations [15]. Control experiments in our laboratory have shown that ethanol does not significantly induce or inhibit neither CYP2C19 nor CYP2E1 activities in cultured human hepatocytes within the ethanol concentration range (up to 1%), as applied in our experiments. Neither was any degradation of the two enzymes found during the experiment, as judged from their

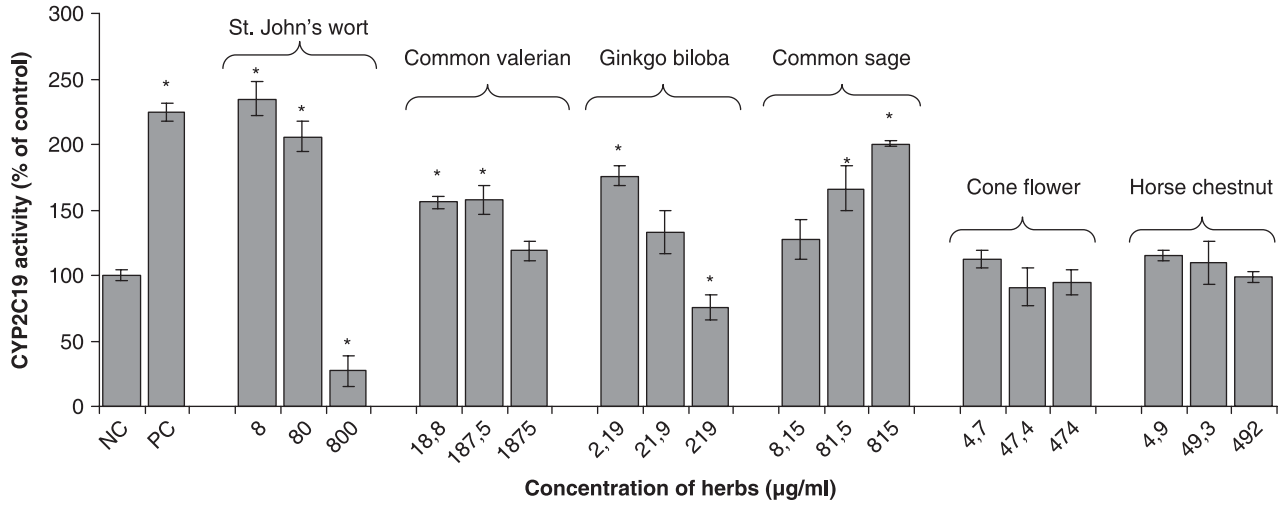

Fig. 1. Human hepatocyte CYP2C19 (S-mephenytoin hydroxylation) activities with: no xenobiotic added, basic control (NC); the positive inducer control rifampicin (PC), 41.2 µg/ml, added or herbal extracts of St John's wort, common valerian, *Ginkgo biloba*, common sage, cone flower or horse chestnut added. All values are given as means ± SD of three replicates. *Statistically different from the basic control ($P < 0.05$).

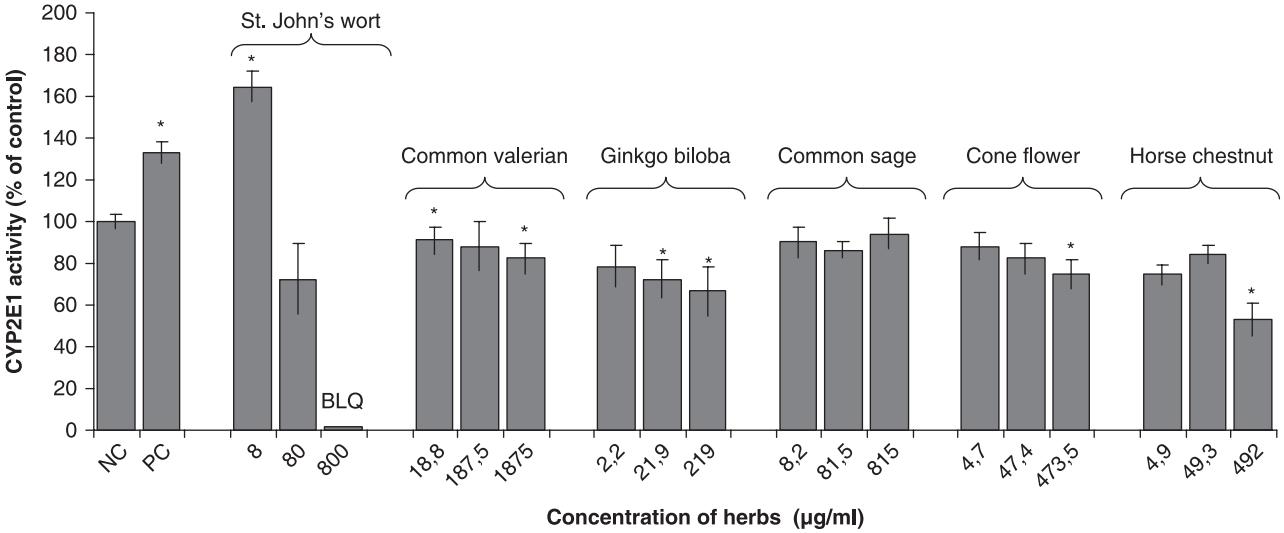

Fig. 2. Human hepatocyte CYP2E1 (chlorzoxazone hydroxylation) activities with: no xenobiotic added, basic control (NC); the positive inducer control rifampicin (PC), 41.2 µg/ml, added or herbal extract of St John's wort, common valerian, *Ginkgo biloba*, common sage, cone flower or horse chestnut added. All values are given as means ± SD of three replicates; BLQ: below limit of quantitation. *Statistically different from the basic control ($P < 0.05$).

unchanged day-to-day basic activities. It has been shown that chlorzoxazone, used in our study as a CYP2E1 substrate, is also metabolized by human liver CYP1A and CYP3A. This contribution was, however, considered to be minor [16] and of no significance for our results.

St John's wort increased the activity of both CYP2C19 and CYP2E1 in our human liver hepatocytes at the lowest concentration, 8 µg/ml, by 134% and 65%, respectively. A strong inhibition of both enzymes was demonstrated at the highest concentration. The increased activity at the low dose is in agreement with previously published results on the ability of St John's wort to induce CYP2C19 and CYP2E1 activities in man. In a study by Wang *et al.* [17], a daily intake of 900 mg St John's wort for 14 days showed a significant induction of CYP2C19 activity by increasing the urine accumulation of 4′-hydroxymephenytoin by 151 ± 92%. In a study by Gurley *et al.* [13], a daily intake of 900 mg St John's wort for 28 days showed an induction of CYP2E1 activity by increasing the 6-hydroxychlorzoxazone/chlorzoxazone serum ratio by nearly 30%. These results are both compatible with a liver induction of CYP2C19 and CYP2E1 by systemic concentrations of St John's wort.

The total systemic plasma concentration of all constituents of St John's wort at steady state is difficult to assess. However, assuming that hyperforin is an acceptable reference substance for most constituents of St John's wort, a total plasma concentration of St John's wort of about 10 µg/ml can be estimated at a recommended dosing of 900 mg daily. This estimation is based on recently published data [13] where a hyperforin steady-state plasma concentration of 51 ± 10 ng/ml (95 nM) was achieved after a recommended dosing of an ethanol extract of St John's wort containing originally hyperforin in an amount of 5.3 mg/g dry weight total herb. According to the manufacturer, our St John's wort batch was comparable to that used by Gurley *et al*. However, a variation in bioavailability among herbal constituents in general can be expected, making some uncertainties in our estimation [18–20]. These estimates indicate that the lowest total concentration of St John's wort used in our hepatocytes, 8 µg/ml, is in the same range as that obtained in plasma with a recommended dosing of St John's wort.

The CYP inhibitions observed at the highest concentration of St John's wort (800 µg/ml, hyperforin concentration 4.2 µg/ml) might in theory be due to cytotoxic effects from hyperforin. In chronic exposure experiments (48 hrs) with purified hyperforin, a limit of 1.3 µg/ml (2.5 µM) has been suggested to be the limit of what hepatocytes can handle of this chemical entity [21]. However, acute exposures with 5 or 10 µM hyperforin produced no cytotoxicity and an inhibition of hepatocyte CYP3A4 activity by approximately 40% and 50%, respectively, was reported. Our hepatocytes were inspected daily by microscopy, and no loss of cell attachment or other indications of cell death were observed during the experiment. Cytotoxicity caused by hyperforin in our more complex total extract of St John's wort was thus considered less probable.

Ginkgo biloba increased CYP2C19 activity in our hepatocytes at the lowest test concentration (2.2 µg/ml) by 75% and decreased CYP2C19 activity by 25% at the highest (219 µg/ml). This is, to our knowledge, the first dose-dependent biphasic effects reported on CYP2C19-mediated metabolism. One human study has indicated an *in vivo* CYP2C19 induction by *G. biloba* [12]. The same study showed that 140 mg *G. biloba* daily, a recommended dosing, for 12 days produced mean steady-state plasma concentrations of the *G. biloba* constituent Ginkgolide A of 7.5–9.7 ng/ml, with peak plasma concentrations

of 32–36 ng/ml. A similar estimate as that made for St John's wort will yield 0.5–0.65 μg/ml 'total-herb' for steady-state and 2.1–2.4 μg/ml for peak plasma concentrations, which are similar to our lowest tested concentration of *G. biloba*. Ginkgolide A content of the *G. biloba* used was 2.1 mg/140 mg total herb.

Common valerian increased CYP2C19 activity by 57% at both of the two lowest concentrations. This may indicate an induction of CYP2C19 also at lower concentrations of common valerian and should thus be especially noted. One investigation in man indicates no inhibition or induction of CYP2D6, 2E1, 3A4, or 1A2 [22], and concludes that significant interactions between common valerian and prescribed drugs depending on these metabolic pathways will most likely not happen. This report is in agreement with our results as far as the lack of CYP2E1 induction is concerned.

Common sage showed a dose-dependent induction of CYP2C19 activity, with a 30–70% increase from 8–82 μg/ml. By using daily recommended doses of common sage (450 mg), such concentrations are easily reached in the small intestine. CYP2C19 exists in the gut wall [5], and previous investigations have suggested that CYP2C19 enzymes in the intestine are capable of presystemic metabolism of drugs like omeprazole and s-mephenytoin. It has been suggested that this presystemic CYP2C19 metabolism might have implications for bioavailability of these drugs and other CYP2C19 substrates [23,24]. Thus, an increased presystemic metabolism of CYP2C19 substrates due to a common sage induction in the small intestine is possible in theory and possible effects on drug bioavailability in man should be investigated.

Only St John's wort increased CYP2E1 activity in this investigation. The lack of herbal effects on CYP2E1 as compared with CYP2C19 may be due to the fact that CYP2E1 induction is mediated through other mechanisms than that of CYP2C19 [25,26].

Due to limited availability and erratic supply of fresh human liver tissue, the hepatocytes used in this study came from only one donor, and thus do not cover any interindividual variation. Furthermore, CYP2C19 is characterised as a highly polymorphic enzyme [27,28]. This certainly has an impact on drug metabolism but will also influence enzyme activity regarding inhibition and induction of the enzyme [17,29]. Ethnic differences in polymorphism rate are also demonstrated [29,30] and results comparing enzyme activity to genotype are variable. The basic enzyme activities achieved in our hepatocytes were, however, within the accepted normal variation of the laboratory, indicating normal enzyme expression of working enzymes (i.e. wild-type). The fact that the induction control and herbal preparations increased CYP2C19 activity also shows that the hepatocytes contained working CYP2C19 enzymes. An analysis of CYP2C19 and CYP2E1 mRNA levels and quantification of protein levels would have been beneficial for the study as a supplement to the measured enzyme activities, especially as only three different herbal concentrations were used.

In conclusion, CYP2C19 was more prone to changes in activity than CYP2E1. St John's wort was the most potent CYP-modulating herb, showing a dose-dependent induction/inhibition effect towards CYP2C19 and possibly also towards CYP2E1, with induction at low dosages and inhibition at higher. Whether cooperative mechanisms are involved or not remains an open question. *G. biloba*, common sage and common valerian all induced CYP2C19 activity in contrast to horse chestnut and cone flower. *Ginkgo biloba* showed an induction/inhibition profile towards CYP2C19 similar to that observed for St John's wort, while common sage showed an increased induction with increasing dosages. Only St John's wort induced CYP2E1 activity.

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