British Journal of Pharmaceutical Research
4(15): 1840-1860, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Potential Interactions of Herbal Extracts of St. John´s Wort with Metabolites of Diazepam in an Organotypical Sandwich Culture of Primary Porcine Hepatocytes

Ali Acikgöz[1,2], Angelika Langsch[3], Augustinus Bader[1] and Shibashish Giri[1*]

[1]University of Leipzig, Center for Biotechnology and Biomedicine, Cell Techniques and Applied Stem Cell Biology, Deutscher Platz 5, 04103 Leipzig, Germany.
[2]Klinikum St. Georg, Delitzscher Straße 141 04129 Leipzig, Germany.
[3]Hannover Medical School, Hannover, Germany.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

Received 19th February 2014
Accepted 9th May 2014
Published 1st August 2014

ABSTRACT

Extracts of St. John's Wort (*Hypericum perforatum*) are known to cause interactions with certain conventional drugs. Herein, we focus on two clinically relevant concepts. First, St. John´s Wort has been used by people of all ages as an herbal treatment for depression without medical prescription. Second, diazepam-like substances called endogenous benzodiazepines are found in cases of acute liver failure without previous exposure to diazepam. Currently diazepam has over 500 brands name and well marketed throughout the world and became one most frequently prescribed medication in different form (oral, injectable, inhalation and rectal forms) for four decades. Based on this concept, we investigated diazepam biotransformation by incubation of a widely accepted in vitro organotypical culture model with *Hypericum* methanolic extract, powdered drug, infusion, oil, and with the pure *Hypericum* compounds hyperforin and hypericin. The amounts of the preparations and compounds were chosen according to the recommended daily medication doses. We measured the activities of ethoxyresorufin-O-deethylase (EROD), ethoxy coumarin *O*-deethylase (ECOD) and the potential induction of diazepam

**Corresponding author: Email: shibashish.giri@bbz.uni-leipzig.de;*

metabolites (desmethyldiazepam, temazepam and oxazepam) during biotransformation. None of the preparations or substances induced EROD or ECOD. However, different preparations did induce the formation of desmethyldiazepam and temazepam. The strongest activity was caused by the extract, followed by the powdered drug and hyperforin. All preparations and compounds increased the formation of the diazepam metabolite oxazepam, but only the extract, the drug powder and the pure compounds had marked effects. Therefore, we report here the potential interference of St. John´s Wort with all three metabolites of diazepam in an organotypical sandwich model that can be utilized to study potential interaction of metabolites of many drugs with herbal ingredients in preclinical stage of drug discovery process.

Keywords: In vitro model; pharmaceutical screening; primary porcine hepatocytes; drug-drug interactions, cytochrome P450; St. John's Wort.

1. INTRODUCTION

Newly developed drugs have to undergo rigorous preclinical safety evaluations. Until recently these have mainly been performed in animal models. Since the predictive power of rodent trials is limited and their ethicality is questioned, there is increasing interest in suitable *in vitro* models. Human hepatocytes are the first choice for the study of hepatic metabolism. However, due to their limited availability larger pharmaceutical screenings are difficult. Hence, alternatives are required that approximate as closely as possible the qualitative and quantitative biotransformation characteristics of human hepatocytes.

Extracts of St. John's Wort are well established as an herbal anti-depressant. In recent years, a number of interactions of St. John's Wort extract have been reported with several co-administered drugs, e.g., oral contraceptives, the anti-coagulants phenprocoumon [1] and warfarin [2], the anti-asthmatic theophylline [3], the immunosuppressant cyclosporin [4,5] and the HIV protease inhibitor indinavir [6]. Clinical trials with healthy volunteers showed a decrease in plasma levels of digoxin [7], phenprocoumon [8], and amitriptylin [9] and a change in the 6-β-OH-cortisol/cortisol ratio in urine [10,11] after oral administration of St. John's Wort. Karliov reported [12] a case report about a drug interaction between cyclosporin A and Hypericum perforatum in orthotopic liver transplantation.

Very interestingly, different types of *Hypericum* preparations, e.g., tea, red oil, expressed juice, powdered herb and alcoholic extract exhibit differing clinical interaction potential [11,13-15]. The predominant importance of the hyperforin content for the clinical interaction potential of a given *Hypericum* preparation is particularly highlighted by the study of Mai et al. [11] who compared a regular methanolic *Hypericum* extract with the same extract after selective removal of hyperforin.

Herein, we focus on two clinically relevant concepts. First, St. John´s Wort has been used by personas of all ages as an herbal treatment for depression without medical prescription. Second, diazepam-like substances called endogenous benzodiazepines are found in cases of acute liver failure without previous exposure to diazepam [16]. Based on this concept, we tested the induction potential of all possible forms of St. John´s Wort in the biotransformation of diazepam by a widely accepted in vitro organotypical culture model. We investigated the screening potential in two different test series with six different St. John's Wort preparations and two compounds. The first series tested a methanolic extract (Jarsin 300®), powdered

Hypericum drug, *Hypericum* infusion and an oil for their potential to interact with CYP 1A (EROD, ethoxyresorufin-O-deethylase), CYP 2B (ECOD, ethoxycoumarin-O-deethylase) and isoenzymes of the CYP 2C and 3A groups that are involved in diazepam metabolism. The known inducers 3-methylcholanthrene (3-MC, inducing EROD), phenobarbital (PB, inducing ECOD) and dexamethasone (DEX, inducing CYP 3A4) were used as positive controls. The second series tested the extract, three different powdered drugs and the compounds hyperforin and hypericin for their effect on diazepam metabolism. Again, DEX was used as the control inducer. Generally, diazepam biotransformation led to the formation of three metabolites (desmethyldiazepam, temazepam, oxazepam) typically found in vivo in humans. Previously, we have reported that drug metabolizing enzymes are highly expressed in an organotypical sandwich model but not in a collagen gel-coated model [17].

2. MATERIALS AND METHODS

2.1 Chemicals

Chemicals were purchased from various sources: $CaCl_2$, glucagon, and KCl were from Merck (Darmstadt, Germany); collagenase, fetal calf serum, and William's Medium E were from Biochrom (Berlin, Germany); L-glutamine, penicillin, and streptomycin were from GIBCO (Eggenstein, Germany); insulin was from Hoechst (Frankfurt, Germany); *Hypericum* preparations, hyperforin, hypericin and Cremophor RH 40 were from PhytoLab (Vestenbergsgreuth, Germany); DMEM was from Cellconcepts, (Umkirch, Germany); and HBSS was from PAA Laboratories (Linz, Austria). All other chemicals were purchased from Sigma (Steinheim, Germany).

2.2 Cell Isolation

The protocol for cell isolation can be found elsewhere or in our previous report [17]. Briefly, female pigs (German Landrace, 6-8 weeks old, body weight 20-30 kg) were obtained from the animal breeding institute at Mariensee, Germany. The animals were hepatectomised and the livers transferred to the lab in sterile cold Ringer buffer. Cells were isolated according to a modification of the methods described by Seglen [18] and Hoogenboom et al. [19]. The liver was perfused at 37ºC with buffer A, containing KCl 6.71mM, NaCl 142mM and HEPES 10mM substituted with EGTA 0.2mM. When the excreting buffer stayed clear, the organ was perfused with the same volume of buffer B (same as buffer A but without EGTA) to wash out the EGTA. Afterwards 500 ml of buffer C, containing KCl 6.71mM, NaCl 66.75mM, HEPES 10mM, glucose 11mM, and collagenase 130kU l^{-1} (type IV CLS), were recirculated through the liver for 5 min followed by addition of 10ml $CaCl_2$ (200mM) and further recirculation for half an hour. The organ was then cut into pieces and placed into sterile 4ºC cold buffer D containing 10% of HBSS (10x), HEPES 10mM, BSA 2.0g l^{-1}, glucose 5.56mM and $CaCl_2$ 2mM. The liver cells were released from the liver, the suspension filtered through a nylon membrane (pore size 100 µm, Jürgens, Hannover, Germany), and then centrifuged at 55g for 5 min. The resulting pellet was resuspended in buffer D and washed three times. The cells were next resuspended in culture medium and counted in a haemocytometer in the presence of trypan blue and NaCl. The mean viability was 89% $\pm$ 3%.

2.3 Organotypical Sandwich Model of Primary Porcine Hepatocytes

Primary porcine hepatocytes were enclosed within two layers of collagen as described in Bader et al. [17]. Briefly, collagen solution was used to moisten 24-well cell culture plates to

form first collagen layer (100 -200 µm) before application of the primary porcine hepatocytes cells. Plates were cultivated with a density of $2.5 \cdot 10^5$ cells per well. The cells were seeded and attached for two hours before medium exchange. Two days later a covering collagen gel layer (100-200µm) of 250µl per well was applied and hardened for one hour, when serum-free medium was added. Cells were cultivated at 37ºC with 5% CO_2 and saturated with water vapor. William's Medium E supplemented with 5% fetal calf serum, 453kU l^{-1} penicillin, 310µM streptomycin, L-glutamine 9mM, prednisolone 1.88 µM, glucagon 4.5nM and insulin 180 U l^{-1} was used as the culture medium for the first two days. After the second collagen gel layer was added, the culture was continued under serum-free conditions for the following cultivation period. Culture medium was changed daily. The overall experiment design of organotypical sandwich model of primary porcine hepatocytes to evaluate the potential interaction of major drug metabolites with diazepam is given in Fig. 1.

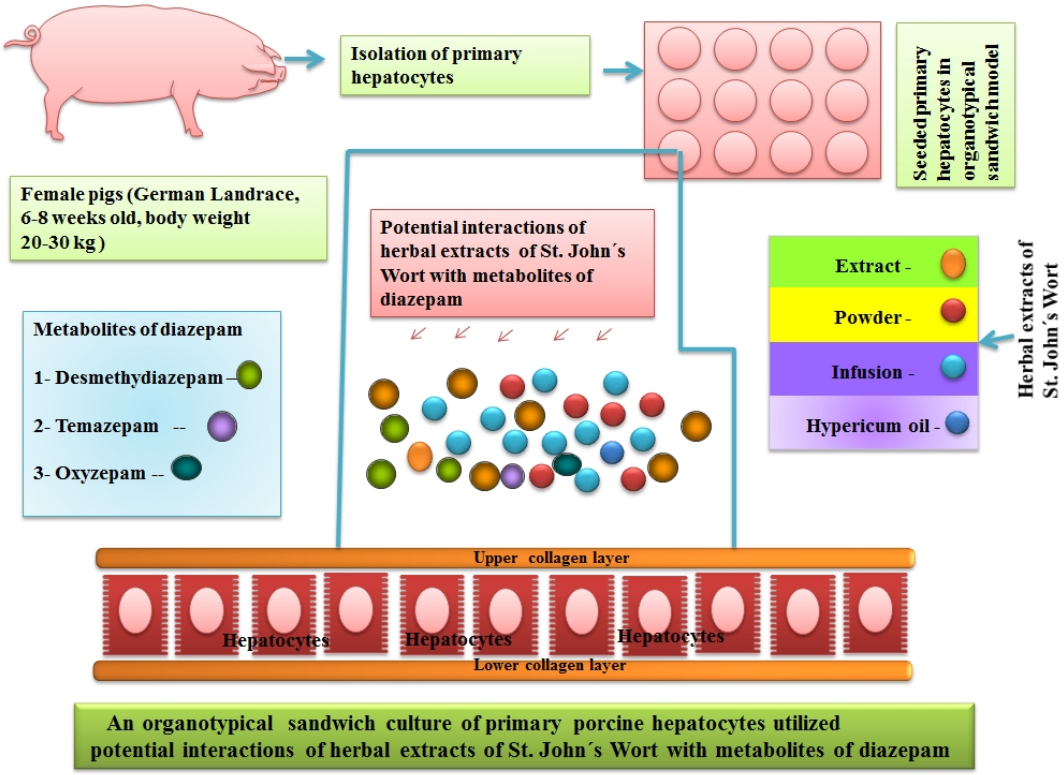


Fig. 1. Organ typical sandwich model of primary porcine hepatocytes to evaluate the potential interaction of major drug metabolites with diazepam

2.4 Preparation of Test Solutions

After the sandwich culture was set up, solutions of the different St. John's Wort preparations and compounds were added. The extract, the powders and the oil were dissolved in 5% cremophor solution for one hour. Cremophor was shown in preliminary analytical trials to dissolve the different classes of *Hypericum* compounds in a concentration representing their relative occurrence in each of the tested preparations. The infusion was prepared with boiling water for ten minutes and adjusted afterwards to 5% cremophor RH 40. Hyperforin

and hypericin were dissolved in DMSO. Three different dilutions of the preparations (1%, 0.1% and 0.01%) and of the compounds (1%, 0.01%, and 0.0001%) were prepared in culture medium. The amounts of the preparations and compounds were chosen according to the recommended daily doses of the corresponding medications (see Table 1).

The concentrations of the *Hypericum* preparations investigated here corresponded to the recommended daily dose of the drug.

Table 1. Concentration of *Hypericum* preparations

Preparation	Input of drug equivalent [mg l^{-1}]
Extract	9000
Powder	3000
Infusion	9000
Hypericum oil	240

To determine the influence of the solvent, the extract was not only dissolved in cremophor RH 40 but also dissolved in DMSO. After incubation for two, three and four days, the cells were thoroughly washed to remove the *Hypericum* preparations. Afterwards, medium containing substrate was added, and the biotransformation activities of the hepatocytes were determined. Model inducers (5µM 3-MC for EROD, 1.5mM PB for ECOD, 200 µM DEX for CYP 3A4) were used to control for the viability and physiological performance of the cells. Furthermore, two solvent controls (Cremophor and DMSO) and a blank control were carried out.

2.5 Assays of Constituents in *Hypericum* Preparations and Test Solutions

The composition of the different *Hypericum* preparations and the respective test solutions (Table 2) was determined by quantitative HPLC methods (PhytoLab GmbH, Vestenbergsgreuth, Germany). Chemical structure of the different St. John's Wort compounds is given in Fig. 2.

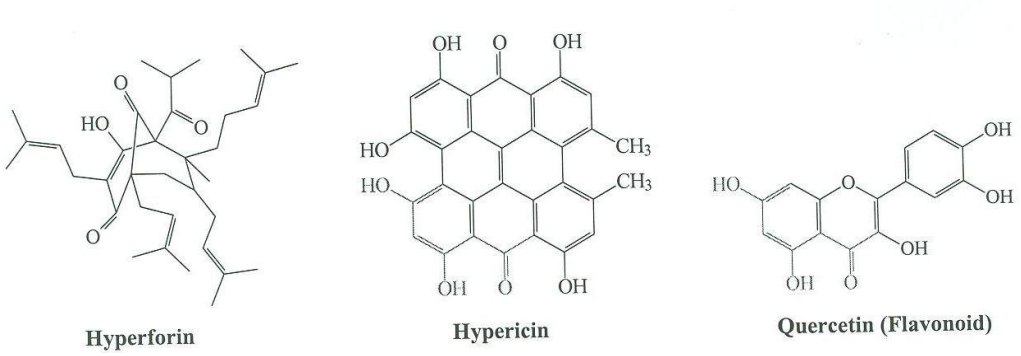


Fig. 2. Chemical structure of the different St. John's Wort compounds

Table 2. Concentration of compounds in culture medium

	Test concentration [%]	ng ml^{-1} culture medium		
		Hyperforin	Hypericin	Flavonoide
Extract (Cremophor)	1	626.8000	32.2000	1928.7000
Extract (Cremophor)	0.1	62.6800	3.2200	192.8700
Extract (Cremophor)	0.01	6.2680	0.3220	19.2870
Extract (DMSO)	1	284.0000	2.5000	2007.0000
Extract (DMSO)	0.1	28.4000	0.2500	200.7000
Extract (DMSO)	0.01	2.8400	0.0250	20.0700
Powder 1	1	9.0000	33.9000	758.0000
Powder 1	0.1	0.9000	3.3900	75.8000
Powder 1	0.01	0.0900	0.3390	7.5800
Powder 2	1	61.2000	12.3000	1437.0000
Powder 2	0.1	6.1200	1.2300	143.7000
Powder 2	0.01	0.6120	0.1230	14.3700
Powder 3	1	50.0000	37.0000	1069.0000
Powder 3	0.1	5.0000	3.7000	106.9000
Powder 3	0.01	0.5000	0.3700	10.6900
Tea	1	8.1000	17.1000	1599.0000
Tea	0.1	0.8100	1.7100	159.9000
Tea	0.01	0.0810	0.1710	15.9900
Oil	1	1.6800	3.8400	1.9000
Oil	0.1	0.1680	0.3840	0.1900
Oil	0.01	0.0168	0.0384	0.0190
Hyperforin	1	100.0000		
Hyperforin	0.01	1.0000		
Hyperforin	0.0001	0.0100		
Hypericin	1		100.0000	
Hypericin	0.01		1.0000	
Hypericin	0.0001		0.0100	

2.6 Ethoxyresorufin-O-Deethylation (EROD) Assay

Deethylation of ethoxyresorufin is CYP 1A-mediated. Hepatocyte cultures were incubated with 50µM of 7-ethoxyresorufin and an equal quantity of dicumarol to prevent further biotransformation of resorufin by cytosolic diaphorase. Aliquots of the supernatant medium were withdrawn after one hour and stored frozen at -20ºC until analysis. After thawing, resorufin conjugates were cleaved using β-glucuronidase 100U ml^{-1} in acetate buffer at 37ºC overnight. Aliquots of the treated samples were mixed with glycine buffer (1.6 M, pH 10.3). Afterwards, formation of resorufin was quantified by fluorometry (LS-5, Perkin-Elmer, Überlingen, Germany) with an excitation wavelength of 530nm and an emission wavelength of 580nm. The spectrofluorometer was calibrated using resorufin standards.

2.7 Ethoxycoumarin-O-Deethylation (ECOD) Assay

Deethylation of ethoxycoumarin is mediated by CYP 2B6. The assay was performed similarly to the EROD assay. Hepatocyte cultures were incubated with 70µM 7-ethoxycoumarin. Aliquots of the medium were withdrawn after one hour and stored frozen at -20ºC until analysis. After thawing, umbelliferone (7-hydroxycoumarin) conjugates were cleaved using 100Uml^{-1} β-glucuronidase in acetate buffer at 37ºC overnight. Aliquots of the treated samples were mixed with glycine buffer. Then, formation of umbelliferone was

quantified by fluorometry with an excitation wavelength of 360nm and an emission wavelength of 460nm. The spectrofluorometer was calibrated using umbelliferone standards.

2.8 Diazepam Biotransformation

Hepatocyte cultures were incubated with 20µg ml^{-1} diazepam. Aliquots of the medium were withdrawn after one hour and stored frozen at -20ºC until analysis. After thawing, the samples were extracted with ethyl acetate and evaporated with nitrogen. Afterwards the substrate and product concentrations were determined by isocratic RP-HPLC. (detection wavelength: 236nm; mobile phase: water/methanol/acetonitrile). Analysis of diazepam and metabolites by HPLC: One microgram of midazolam (as internal standard) and 20µl 4M NaOH were added to 1ml of each probe. After adding 100µl isopropanol, probes were extracted after 30 min with 5ml ethyl acetate and centrifuged 10 min at 200g. The ethyl acetate phase was evaporated under nitrogen atmosphere and the remnant was dissolved in 120µl of the HPLC mobile phase, consisting of acetonitrile+ methanol+0.04% triethylamine (40+10+50 v/v) pH 7. Of this, 80µl was loaded onto an HPLC Nucleosil®-100-5 C18 HD-column from Macherey-Nagel, Germany. The HPLC equipment was from Merck and Hitachi and consisted of an L7100 HPLC pump, an L7200 autosampler, an L7450 UV detector, a D7000 interface module and an HPLC system manager running on a Compaq computer. The flow rate of the mobile phase was 0.8ml min−1 and the column temperature was 22ºC. For the quantification of temazepam, desmethyldiazepam and oxazepam standards of 10ng to 10µg were extracted and measured as described above.

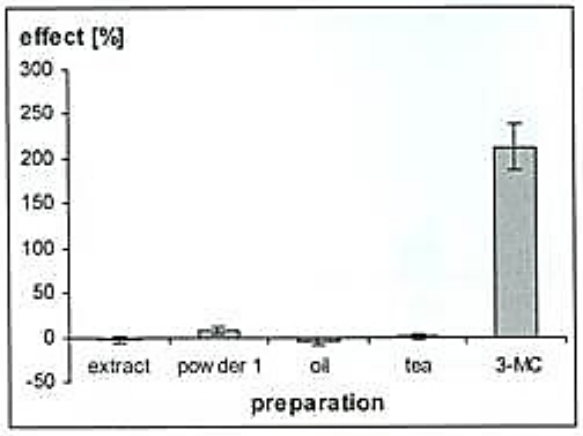
2.9 Statistics

The enzyme activities measured here were transformed into relative values using the corresponding solvent control. To better illustrate the average interaction potential of a preparation or compound, the mean values were taken of the single effects (n = 3) on each metabolite referring to all concentrations and induction periods. Results in tables and figures are expressed as means ± SD. Student's *t*-tests were used to assess the statistical relevance of the all acquired data. Values of *p* smaller than 0.05 were considered statistically significant.

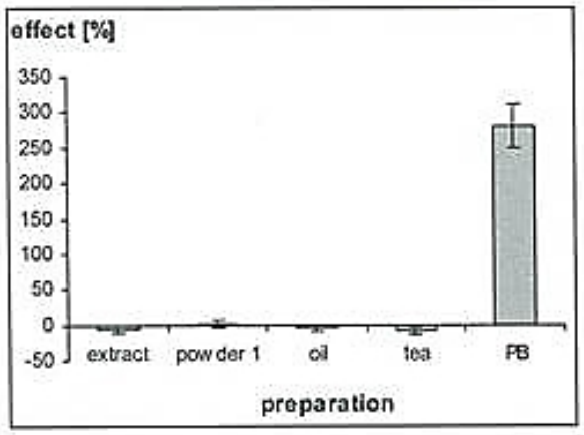
3. RESULTS AND DISCUSSION

3.1 EROD (CYP 1A) and ECOD (CYP 2B)

EROD is an important marker of cytochrome P450 1A activities in hepatic microsomes of pigs [20]. Four different *Hypericum* preparations were assayed for their influence on drug metabolism by CYP 1A and 2B: extract (80% MeOH), powder 1, infusion, and oil. After treatment with St. John's Wort, the activities of both EROD and ECOD were found to be within the same range as the solvent controls. None of the tested preparations had a significant effect regardless of the concentration or duration of treatment (Fig. 1). However, the corresponding model inducers 3-MC and PB induced an increase of 213% and 280%, respectively, in enzyme activity. Neither EROD and ECOD were affected by the St. John's Wort preparations (Fig. 3). A positive effect means an induction of the enzyme's activity, whereas a negative effect means an inhibition of the activity. None of the investigated preparations showed a significant effect on ECOD or EROD. Only the model inducers 3-Methylcholanthrene (3-MC) and phenobarbital (PB) were able to induce the enzymes.



A: Effect on EROD



B: Effect on ECOD

Fig. 3. Induction potential of four different St. John´s wort preparations on EROD (A) and ECOD (B)

3.2 Diazepam Metabolism (CYP 2B6, 2C and 3A)

Diazepam is metabolized by multiple CYP isoenzymes: CYP 2B6, 2C8, 2C9, 2C19, 3A4 and 3A5 demethylate diazepam to form the intermediate product desmethyldiazepam. Diazepam is hydroxylated via an alternative pathway to temazepam by CYP 3A4, 3A5 and 2C19. Both intermediate products are further metabolized to form the end product oxazepam by the corresponding concurrence reaction [21,22]. Hence, three different products must be regarded in diazepam metabolism: Desmethyldiazepam, temazepam and oxazepam. In the first series, the same four *Hypericum* preparations were investigated for EROD and ECOD activity. The second series tested the extract dissolved in DMSO, two more drug powders, and the compounds hyperforin and hypericin.

3.2.1 Desmethyldiazepam

This intermediate product is formed by N-demethylation of diazepam. Fig. 3 shows the average proportional effects of each preparation and constituent on desmethyldiazepam formation. The data of the first series show a strong inductive effect of the extract on all of the liver cell isolates. Furthermore, induction was distinctly dose-dependent (Fig. 4A): higher concentrations of the extract had stronger effects. Powder 1 and the infusion also had an inducing effect on desmethyldiazepam formation, but this was considerably weaker. *Hypericum* oil had no effect. Except for hypericin and the oil, both of which showed a weak but negligible inhibiting influence, all preparations induced the formation of desmethyldiazepam. Remarkably, the extract induced metabolism when dissolved in cremophor RH 40 but inhibited it when dissolved in DMSO (see Table I). The highest induction was reached with the model inducer DEX.

In the second series, the extract dissolved in cremophor again showed the strongest inductive effect. Dissolved in DMSO, it had an inhibitory effect. Hyperforin induced the formation of desmethyldiazepam (Fig. 4B). In contrast, hypericin had a weak inhibiting effect. Overall, the strongest effects were observed for all preparations after two days of treatment (Fig. 4B).

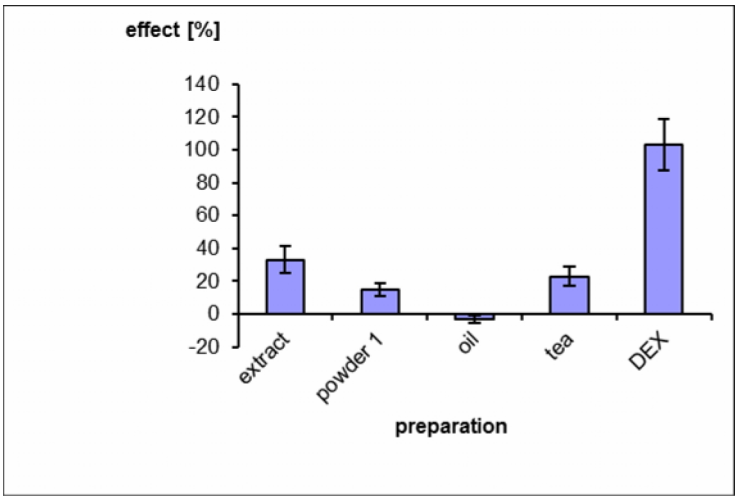
2.2.2 Temazepam

This intermediate product is formed by hydroxylation of diazepam. Fig. 5 shows the average proportional effects of each preparation and constituent on temazepam formation. As already seen in the results for the metabolite desmethyldiazepam, the extract had the strongest inducing effect. Here too, there was a clear dose-dependence. Powder 1 and the infusion had an inducing but weaker influence on this metabolite. Again, *Hypericum* oil did not show any inducing effect (Fig. 5A).

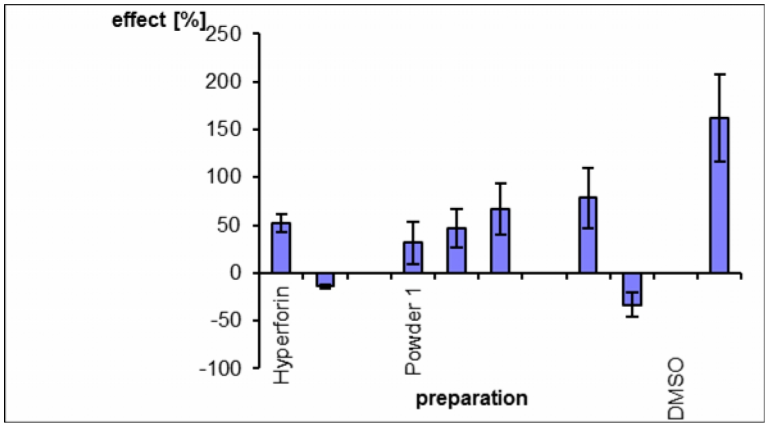
The second series showed that the inducing effect of powders 2 and 3 on temazepam was similar to that of the extract. The influence of powder 1 was distinctly lower. However, the effects of the extract and of powders 1 and 2 were markedly dose-dependent; the extract dissolved in DMSO had an inhibitory effect. Although hyperforin had a dose-dependent inducing effect, hypericin inhibited the formation of temazepam (Fig. 5B). The strongest effects were observed after two days of *Hypericum* treatment. In summary, the investigated preparations and compounds had very similar effects on both intermediate products of diazepam metabolism. Hypericin had a clear inhibiting influence on this metabolite, while the influence of the oil was weak and negligible. All the other preparations were able to induce the formation of temazepam. Additionally, the extract induced metabolism when dissolved in cremophor RH 40 but inhibited metabolism when dissolved in DMSO (see Table I). DEX was used as a model inducer.

2.2.3 Oxazepam

This metabolite represents the end product of diazepam metabolism. It is formed either by hydroxylation of desmethyldiazepam or by N-demethylation of temazepam. Fig. 5 shows the average proportional effects of each preparation and constituent on oxazepam formation. All isolations showed a very strong induction of CYP 3A by DEX. The extract and powder 1 had the strongest inducing effects on oxazepam formation (Fig. 4). The *Hypericum* infusion had about half of this induction potential, and even the oil exhibited a low inducing effect on the formation of this metabolite.



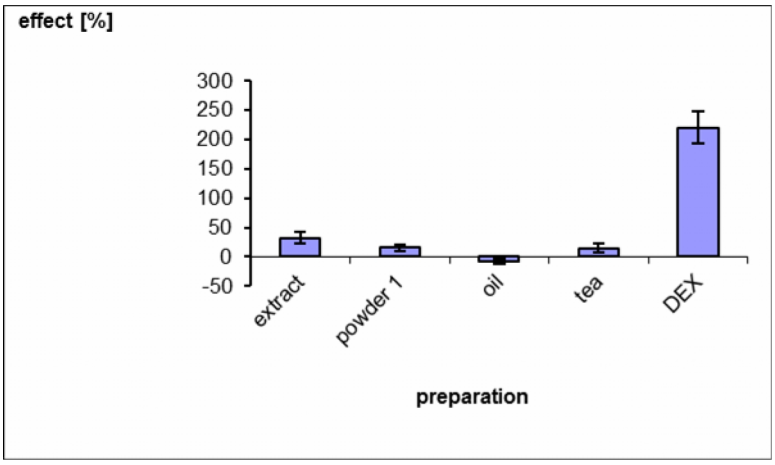
A: First series



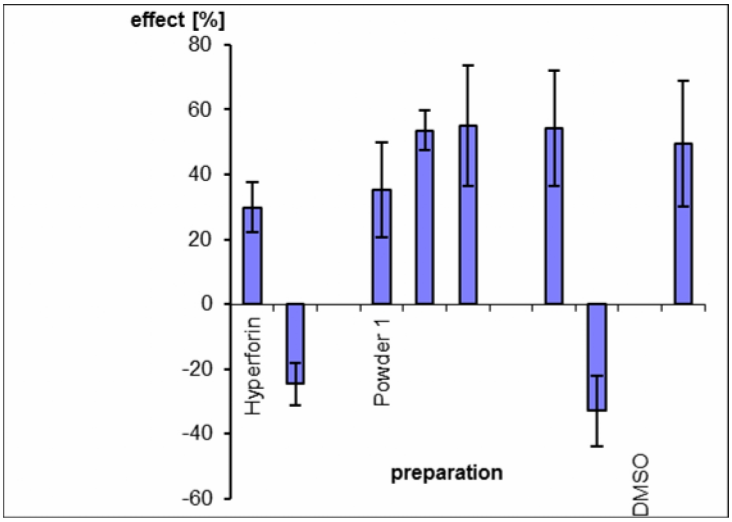
B: Second series

Fig. 4. Induction potential of different St. John's Wort preparations and compounds on the diazepam metabolite desmethyldiazepam

The second series revealed that the compounds hypericin and hyperforin in particular exhibited considerable dose-dependent induction. Powder 3 had the strongest inducing effect on this metabolite. Induction of the formation of oxazepam by powders 2 and 1 was weaker. As with the other metabolites, the extract had an inhibitory effect when dissolved in DMSO. Again, the strongest effects were observed after two days of *Hypericum* treatment (Fig. 6). Overall, there was generally good agreement with regard to the effects of the different preparations and compounds as measured in the two series with the different metabolites. All preparations and compounds were capable of inducing the formation of oxazepam. Again, the effect of the extract depended on the solvent. When the extract was dissolved in cremophor RH 40 metabolism was induced, but when dissolved in DMSO metabolism was inhibited. The model inducer DEX had a very strong inducing effect on oxazepam.



A: First series



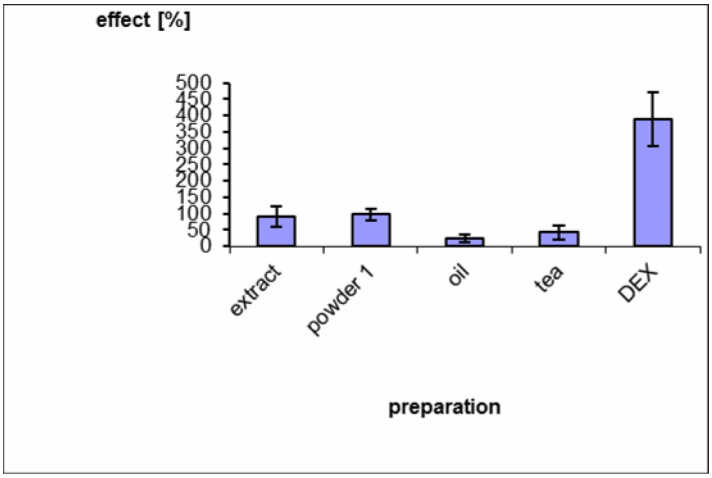
B: Second series

Fig. 5. Induction potential of different St. John's Wort preparations and compounds on the diazepam metabolite temazepam

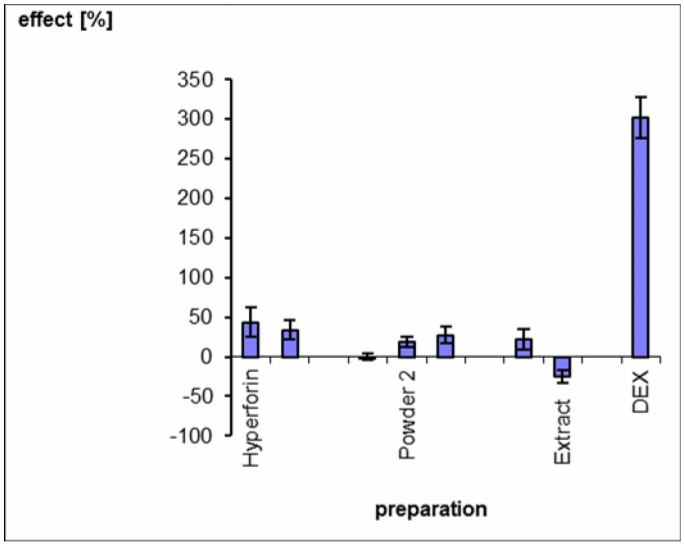
2.3 Induction Concentration and Period

The experiments were performed with three different *Hypericum* concentrations and three different incubation times. In almost all cases, the highest concentration of a preparation and constituent caused not only the highest metabolite concentration but also the maximum effect. The effects of the extract, powder 1 and hyperforin were distinctly dose-dependent, as shown in Fig. 7. Although the highest metabolite concentration was found after three days of treatment, the strongest effects in the corresponding untreated controls were observed after two days of *Hypericum* application. The experiments were carried out with three different *Hypericum* concentrations and for three different periods of time. In almost all cases, the

highest concentration of a preparation and constituent caused the highest metabolite concentration. The effects of extract, powder 1 and hyperforin in particular were markedly dose-dependent.

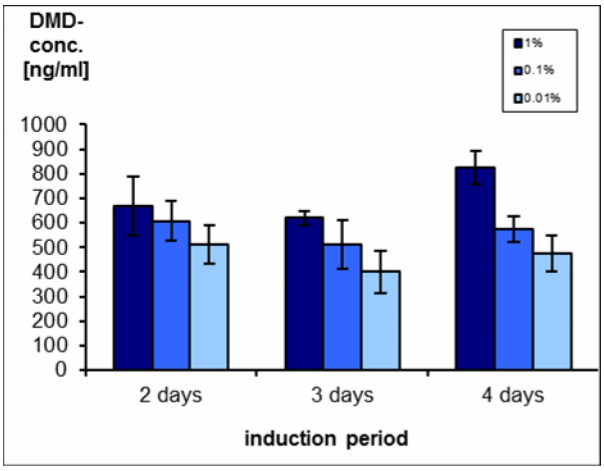


A: First series

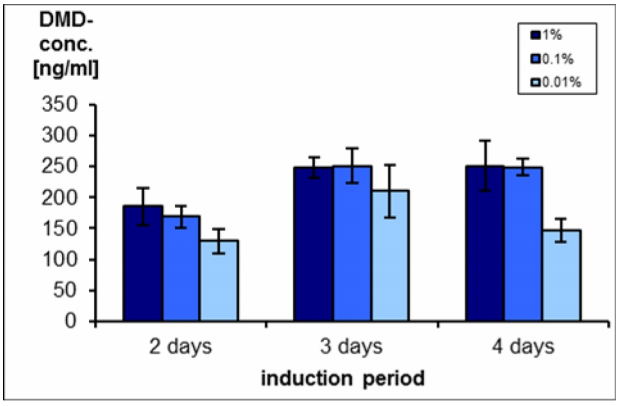


B: Second series

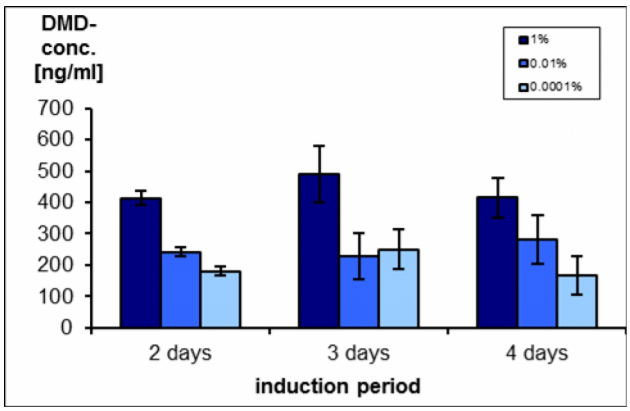
Fig. 6. Induction potential of different St. John's Wort preparations and compounds on the diazepam metabolite oxazepam



A: Extract



B: Powder 1



C: Hyperforin

Fig. 7. Dose-dependent changes in desmethyldiazepam (DMD) concentration by the extract, powder 1 and hyperforin

When a patient takes medicine, food and beverages may often be taken with the medication. Mostly patients consume tea without a physician's prescription. However, this increases the chances of interactions with the prescribed drug that could change the pharmacokinetic profile, block the drug's efficacy or even create toxicity. Ruschitzka et al. [5] reported acute heart transplant rejection due to St. John's wort. Interestingly, unwanted pregnancy has been associated with the use of St John's wort in women taking oral contraceptives [23]. In addition, St. John's Wort has potential to interact with a number of drugs such as Theophylline [3], phenoprocumon [8], Dioxin [7], HIV protease inhibitors [6], and selective serotonin re-uptake inhibitors [24]. Mills et al. [25] determined the methodological quality of clinical trials that examined possible interactions of St John's wort with conventional drugs, and they gave a safety suggestion that clinicians and patients should beware of possible decreases in the systemic bioavailability of conventional drugs when taken concomitantly with St John's wort.

Over the past decade, toxicological research on the interactions between St John's wort and drugs has increased. Herein, we investigated the induction by St John's wort of diazepam biotransformation in an in vitro organotypical cellular model. Diazepam is a type of sedative-hypnotic drug called a benzodiazepine. It has anxiety-relieving effects and it has been used worldwide for approximately forty years to treat a wide range of conditions such as insomnia, alcohol withdrawal, epilepsy and other neuropsychiatric disorders, and it is routinely used in other surgical arenas. However, benzodiazepine is associated with hepatic encephalopathy, which is possibly cause by endogenous benzodiazepine and elevated ammonia levels. Basile et al. [16] detected diazepam in patients suffering from acute liver failure with hepatic encephalopathy without previous exposure to diazepam. Basically, the liver and the brain interact in numerous ways to ensure normal brain functioning. St John's wort is the seventh biggest botanical supplement [26], and in some countries like Germany it is commonly prescribed for mild treatments, especially in children and adolescents [27]. Furthermore, it has been used for more than 200 years and 111 million daily doses of hypericum extract have been taken in Germany alone.

The World Health Organization reports that 80% of people worldwide rely on herbal medicines for some aspect of their primary health care. In the last 20 years in the United States, the use of herbal medicine has increased along with dissatisfaction with the cost of prescription medications. In Germany, roughly 600 - 700 plant-based medicines are currently available and are prescribed by approximately 70% of German physicians. About 60 million Americans (1 in 5) use complementary and alternative therapy, and it was estimated that 20% of patients regularly taking prescription drugs were also taking herbal medicine, which suggests that about 15 million Americans are at potential risk for herb-drug interactions. Also, about a third of patients reported they seek complementary and alternative therapies for health promotion and disease prevention [28,29]. Some herbal medicines may cancel the effect of a prescription drug; others may reduce it, or even exaggerate it. So it is clinically essential to at least screen for the most commonly used daily herbal supplements. We hypothesize based on this study that in vitro organotypical sandwich models will facilitate the easy and rapid screening of a wide range of herbal –drug interactions.

None of the *Hypericum* preparations and compounds studied here affected CYP 1A (EROD) or CYP 2B (ECOD) activity. Most of the preparations did affect CYP 2C and 3A, but there were clear differences between the preparations. The perceptible tendencies of the different preparations confirmed in regard to the cytochrome P450-dependent metabolism of diazepam corresponded very well between the two intermediate products

desmethyldiazepam and temazepam. A similar hierarchy was also found for the end product oxazepam. Only the constituent hypericin differed in its influence on the metabolites.

Most remarkable were the different influences of the extract. When dissolved in cremophor RH 40, the extract generally showed the highest induction potential of all tested preparations. In contrast, when dissolved in DMSO it consistently inhibited the formation of the different metabolites. Analysis of the two extract solutions revealed that mainly the flavonoids were dissolved in DMSO, while the more lipophilic compounds hyperforin and hypericin were not found in the expected amounts. In the cremophor solution, however, the relative concentrations of all the analyzed compounds corresponded to those of the original extract; this was also the case for the other preparations. Table 2 displays the concentrations (measured by PhytoLab GmbH & Co. KG, Vestenbergsgreuth) of the different compounds of the investigated *Hypericum* preparations in culture medium. These results suggest that the specific composition of a St. John's Wort preparation plays an important role in its overall interaction potential and that hyperforin may mainly be responsible for the observed effects. Although there was also induction of oxazepam formation by hypericin, it seems unlikely that this compound contributes to interactions *in vivo* due to its relatively low concentration in *Hypericum* products as compared with the hyperforin content of *Hypericum* extracts.

The results of Moore et al. [30] support these findings. Three different St. John's Wort extract preparations and pure hyperforin were found to induce CYP 3A4 in human hepatocytes, while hypericin and different flavonoids appeared primarily inactive. Roby et al. [11] found induction of CYP 3A by St. John's Wort *in vivo*. Bioavailability data for hyperforin, hypericin and other *Hypericum* compounds have been published by Biber et al. [31] and flavonoids are well known to be only poorly absorbed in the intestines.

In contrast, our data suggest that the effect of flavonoids is inhibition. Markowitz et al. [32] make a similar assumption. Flavonoids are well known to have an inhibiting effect on the human CYPs 3A4, 1A2, 2B and 2E1. Quercetin in particular, one of the compounds of St. John's Wort, is considered to be a potent inhibitor of CYP 3A4 [32] and 1A2 [33]. However, flavonoids do not seem to be of any importance for the clinical interaction potential of *Hypericum*. *Hypericum* tea, which has a high flavonoid content but is practically devoid of hyperforin [14] and a hyperforin-free methanolic extract had no significant clinical effect on the pharmacokinetics of Digoxin [14] or Cyclosporin [13].

The interaction profile that is the most well documented by case reports, clinical observations and volunteer studies is that of the St. John's Wort preparation "Jarsin 300®," which was also the methanolic extract used in our studies. The published data [1-3, 5-10] indicate that it has an inductive effect on the metabolism of multiple drugs, which is in good accordance with our results. Moreover, our data are congruent with those of Moore et al. [30] and Wentworth et al. [34], who found the constituent hyperforin to be a highly potent activator of the pregnane X receptor (PXR), an orphan nuclear receptor known to induce hepatic CYP 3A gene expression in response to xenobiotics [35,36]. In contrast, hardly any PXR activity was found for hypericin or *Hypericum* flavonoids. These results explain the effects of the particular constituents found in our research.

Our investigations show that *in vitro* studies with primary porcine hepatocytes are a valuable tool for the detection of drug-drug interactions. This not only applies to isolated compounds but also to complex herbal drug preparations. Zhao et al. [37] investigated the applicability of different *in vitro* systems (mouse, rat and dog microsomes) in comparison to human

microsomes. They found that the models phylogenetically closest to man are both qualitatively and quantitatively the most suitable for screening for interactions. The best results were obtained with canine microsomes, and the least suitable were the results obtained with murine microsomes. *In vitro* models offer the advantage of reduced complexity of the study system and the ability to evaluate intrinsic metabolic potentials and mechanisms with respect to a specific reaction [38]. These data are essential for understanding and predicting drug interactions.

Some reports have indicated that the organotypical sandwich culture model facilitates the preservation of certain liver characteristics, including cuboidal morphology of hepatocytes with features such as bile canaliculi, tight junctions, and gap junctions [39-44]. In a previous study, we used a modified sandwich technique and corresponding hormonal culture medium composition that provided the basis for a demonstration that this model correctly reflected the species dependence of their in vivo metabolism of the antihypertensive drug urapidil and its metabolites [45], suggesting that this model is the best for the prediction of the metabolism of xenobiotics. Furthermore, we have already reported on the maintenance of phase I and phase II metabolic activity of ethoxyresorufin, ethoxycoumarin and testosterone metabolism in rat and human hepatocytes over a period of two weeks at a level comparable to freshly isolated cells [46]. The expression of CYP211 enzymes is generally lost in cell culture, but we found in our previous study higher metabolites in our organotypical model than in freshly isolated cells [46]. This indicates that there are many possible ways of obtaining higher expression of other cytochrome P450 enzymes in an organotypical model. This is a very important fundamental point for further investigation regarding drug biotransformation as well as drug–drug interactions for better and safer drug production as well as for reducing post-market drug withdrawals. Further research is needed to know the full potential of St. John's Wort to interact with drugs. However, in vitro organotypical sandwich models are likely to serve as valuable platforms for identifying drug-herbal interactions and other potential toxic effects of a wide range of herbal materials for both short-term and long-term observation.

As pigs are phylogenetically closely related to humans, porcine hepatocytes could represent a suitable and more accessible model for *in vitro* screening of drug interactions. Recently, we evaluated the interspecies differences of rat, porcine, and human hepatocytes on the basis of liver-specific functions and biotransformation of testosterone in an organotypical sandwich model and found that porcine hepatocytes are much closer to primary human hepatocytes [47].

The use of conventional in vitro collagen-coated hepatocyte cultures has been strongly criticized both for toxicity studies and preclinical drug development because cultured hepatocyte rapidly lose liver-specific functions in monolayer culture. Furthermore, pharmacological and toxicological studies of the biotransformation of drugs require that cell culture models express the appropriate drug-metabolizing enzymes. Therefore, we adapted the modified sandwich liver culture model [48] to porcine cells, originally based on Dunn et al. [49,50]. Cells were cultivated as a coculture of parenchymal and nonparenchymal liver cells in a 3D configuration in the modified sandwich model [48]. This technique provides an adequate microenvironment for the restabilization of the architecture of the liver cells and provides a morphological reorganization that corresponds closely to that observed *in vivo*. With this technique, liver-specific functions can be maintained for several weeks. This is crucial for kinetic and toxicological studies on the biotransformation of different pharmaceuticals [51]. However, there have been few studies evaluating the collagen sandwich as a system for measuring herbal drug interactions. Here we used the most

accepted organotypical model (based on our previous report [52] which is a form of the sandwich model modified for better hepatic liver functions than conventional culture, including biotransformation [44,45,47,52]. For this reason, we used the organotypical model for this study.

4. CONCLUSIONS

Our results show interference of St. John´s Wort with all three of these major metabolites of diazepam in such an organotypical sandwich hepatocytes model. St. John´s Wort induce the formation of desmethyldiazepam and temazepam. The strongest activity was caused by the extract, followed by the powdered drug and hyperforin. However, *in vitro* organotypical sandwich models are likely to serve as valuable platforms for identifying drug-herbal interactions and other potential toxic effects of a wide range of herbal materials for both short-term and long-term observation. It was found that the constituent hyperforin to be a highly potent activator of the pregnane X receptor (PXR), an orphan nuclear receptor known to induce hepatic CYP 3A gene expression in response to xenobiotics. In contrast, hardly any PXR activity was found for hypericin or *Hypericum* flavonoids. These results explain the effects of the particular constituents found in our research. *In vitro* models offer the advantage of reduced complexity of the study system and the ability to evaluate intrinsic metabolic potentials and mechanisms with respect to a specific reaction. These data are essential for understanding and predicting drug interactions. Our investigations show that *in vitro* studies with primary porcine hepatocytes are a valuable tool for the detection of drug-drug interactions. This not only applies to isolated compounds but also to complex herbal drug preparations. This porcine models phylogenetically closest to man are both qualitatively and quantitatively the most suitable for screening for interactions.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

ACKNOWLEDGEMENTS

This work was financially supported by Bad Heilbrunner Reform-Diät-Arznei GmbH & Co.; Börner GmbH; Diapharm; M.C.M. Klosterfrau Köln; Kneipp-Werke; Merz + Co. GmbH & Co.; Salus Haus; Jenapharm GmbH & Co. KG; Astrid Twardy GmbH; Alsitan W.E. Ronneburg GmbH; Martin Bauer GmbH & Co. KG; Isis Puren Arzneimittel GmbH & Co. KG; Bioforce AG; H + S Tee-Gesellschaft; Dr. Kolkmann & Partner GmbH; Bionorica Arzneimittel GmbH; Sidroga Schweiz; Bombastus-Werke GmbH

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Bon S, Hartmann K, Kuhn M. Johanniskraut: Ein Enzyminduktor? Journal suisse de pharmacie. 1999;16:535-536.
2. Yue QY, Bergquist C, Gerden B. Safety of St John's wort (*Hypericum perforatum*). Lancet. 2000;355(9203):576-7.
3. Nebel A, Schneider BJ, Baker RK, Kroll DJ. Potential metabolic interaction between St. John's wort and theophylline. The Annals of pharmacotherapy. 1999:33(4):502.
4. Ruschitzka F, Meier PJ, Turina M, Luscher TF, Noll G. Acute heart transplant rejection due to Saint John's wort. Lancet. 2000;355(9203):548-9.
5. Piscitelli SC, Burstein AH, Chaitt D, Alfaro RM, Falloon J. Indinavir concentrations and St John's wort. Lancet .2000;355(9203):547-8.
6. Johne A, Brockmoller J, Bauer S, Maurer A, Langheinrich M, Roots I. Pharmacokinetic interaction of digoxin with an herbal extract from St John's wort (*Hypericum perforatum*). Clinical pharmacology and therapeutics. 1999;66(4):338-45.
7. Maurer A, Johne A, Bauer S, Brockmöller J, Donath F, Roots I, Langheinrich M, Hübner, WD. Interaction of St Johns wort extract with phenoprocumon. Eur J Clin Pharmacol. 1999;55:A22.
8. De Smet PA, Touw DJ. Safety of St John's wort (*Hypericum perforatum*). Lancet. 2000;355(9203):575-6.
9. Kerb R, Bauer S, Brockmöller J, Roots I. Urinary 6-β-hydroxycortisol excretion rate is affected by treatment with Hypericum extract. Eur J Clin Pharmacol. 1997;52:A186(abstr. 607).
10. Roby CA, Anderson GD, Kantor E, Dryer DA, Burstein AH. St John's Wort: Effect on CYP3A4 activity. Clinical pharmacology and therapeutics. 2000;67(5):451-7.
11. Mai I, Bauer S, Perloff ES, Johne A, Uehleke B, Frank B, Budde K, Roots I. Hyperforin content determines the magnitude of the St John's wort-cyclosporine drug interaction. Clinical pharmacology and therapeutics. 2004;76(4):330-40.
12. Karliov,a M, Treichel U, Malagò M, Frilling A, Gerken G, Broelsch CE. Interaction of Hypericum perforatum (St. John's wort) with cyclosporin a metabolism in a patient after liver transplantation. J Hepatol . 2000;3(5):853-855.
13. Mueller SC, Uehleke B, Woehling H, Petzsch M, Majcher-Peszynska J, Hehl EM, Sievers H, Frank B, Riethling AK, Drewelow B. Effect of St John's wort dose and preparations on the pharmacokinetics of digoxin. Clinical pharmacology and therapeutics. 2004;75(6):546-57.
14. Mueller SC, Majcher-Peszynska J, Uehleke B, Klammt S, Mundkowski RG, Miekisch W, Sievers H, Bauer S, Frank B, Kundt G, Drewelow B. The extent of induction of CYP3A by St. John's wort varies among products and is linked to hyperforin dose. European journal of clinical pharmacology. 2006;62(1):29-36.
15. Butterweck V, Schmidt M. St. John's wort: Role of active compounds for its mechanism of action and efficacy. Wien Med Wochenschr. 2007;157(13-14):356-3561
16. Basile AS, Hughes RD, Harrison PM, Murata Y, Pannell L, Jones EA, Williams R, Skolnick P. Elevated brain concentrations of 1,4-benzodiazepines in fulminant hepatic failure. The New England journal of medicine. 1991;325(7):473-8.
17. Maringka M, Giri S, Bader A. Preclinical characterization of primary porcine hepatocytes in a clinically relevant flat membrane bioreactor. Biomaterials. 2010;31(1):156-72.
18. Seglen PO. Preparation of isolated rat liver cells. Methods in cell biology. 1976;13:29-83.

19. Hoogenboom LA, Pastoor FJ, Clous WE, Hesse SE, Kuiper HA. The use of porcine hepatocytes for biotransformation studies of veterinary drugs. Xenobiotica; The fate of foreign compounds in biological systems. 1989;19(11):1207-19.
20. Zamaratskaia G, Zlabek V. EROD and MROD as Markers of Cytochrome P450 1A Activities in Hepatic Microsomes from Entire and Castrated Male Pigs. Sensors (Basel). 2009;9(3):2134-47.
21. Ono S, Hatanaka T, Miyazawa S, Tsutsui M, Aoyama T, Gonzalez FJ, Satoh T. Human liver microsomal diazepam metabolism using cDNA-expressed cytochrome P450s: Role of CYP2B6, 2C19 and the 3A subfamily. Xenobiotica; The fate of foreign compounds in biological systems. 1996;26(11):1155-66.
22. Yang TJ, Shou M, Korzekwa KR, Gonzalez FJ, Gelboin HV, Yang SK. Role of cDNA-expressed human cytochromes P450 in the metabolism of diazepam. Biochemical pharmacology. 1998;55(6):889-96.
23. Schwarz UI, Buschel B, Kirch W. Unwanted pregnancy on self-medication with St John's wort despite hormonal contraception. British journal of clinical pharmacology 2003;55(1):112-3.
24. Gordon JB. SSRIs and St. John's Wort: Possible toxicity? American family physician 1998;57(5):950,953.
25. Mills E, Montori VM, Wu P, Gallicano K, Clarke M, Guyatt G. Interaction of St John's wort with conventional drugs: systematic review of clinical trials. BMJ 2004,. 3,27-30.
26. Blumenthal, M. Herbs continue slide in mainstream market: Sales down 14 percent. Herbalgram. 2003;58:71.
27. Fegert JM, Kolch M, Zito JM, Glaeske G, Janhsen K. Antidepressant use in children and adolescents in Germany. Journal of child and adolescent psychopharmacology. 2006;16(1-2):197-206.
28. Eisenberg DM, Kessler RC, Foster C, Norlock FE, Calkins DR, Delbanco TL. Unconventional medicine in the United States. Prevalence, costs, and patterns of use. The New England journal of medicine. 1993;328(4):246-52.
29. Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, Kessler R. C. Trends in alternative medicine use in the United States, 1990-1997: Results of a follow-up national survey. JAMA: The journal of the American Medical Association. 1998;280(18):1569-75
30. Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins J. L, Kliewer SA. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(13):7500-2.
31. Biber A, Fischer H, Romer A, Chatterjee SS. Oral bioavailability of hyperforin from hypericum extracts in rats and human volunteers. Pharmacopsychiatry. 1998;31Suppl 1:36-43.
32. Markowitz JS, DeVane CL, Boulton DW, Carson SW, Nahas Z, Risch SC. Effect of St. John's wort (*Hypericum perforatum*) on cytochrome P-450 2D6 and 3A4 activity in healthy volunteers. Life sciences. 2000;66(9):PL133-9.
33. Johne A, Schmider J, Maurer A, Brockmöller J, Mai I, Donath F, Roots I. Arzneimittel interaktionen mit Johanniskrautextrakt. In: Rietbrock N (Ed.), Phytopharmaka VI: Forschung und klinische Anwendung, Steinkopff Verlag, Darmstadt. 2000;55-66.
34. Wentworth JM, Agostini M, Love J, Schwabe JW, Chatterjee VK. St John's wort, a herbal antidepressant, activates the steroid X receptor. The Journal of endocrinology. 2000;166(3):R11-6.

35. Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. The Journal of clinical investigation. 1998;102(5):1016-23.
36. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam, A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(21)12208-13.
37. Zhao XJ, Ishizaki T. The In vitro hepatic metabolism of quinine in mice, rats and dogs: Comparison with human liver microsomes. The Journal of pharmacology and experimental therapeutics. 1997;283(3):1168-76.
38. Chiu SH. The use of in vitro metabolism studies in the understanding of new drugs. Journal of pharmacological and toxicological methods. 1993;29(2):77-83.
39. Hoffmaster KA, Turncliff RZ, LeCluyse EL, Kim RB, Meier PJ, Brouwer KL. P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes: Relevance to the hepatobiliary disposition of a model opioid peptide. Pharm. Res. 2004;21:1294–1302.
40. LeCluyse E, Madan A, Hamilton G, Carroll K, DeHaan R, Parkinson A. Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. Journal of biochemical and molecular toxicology. 2000;14(4):177-88.
41. Moghe PV, Berthiaume F, Ezzell RM, Toner M, Tompkins RG, Yarmush ML. Culture matrix configuration and composition in the maintenance of hepatocyte polarity and function. Biomaterials. 1996;17:373–385.
42. Richert L, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P, LeCluyse E. Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. Toxicol. In Vitro. 2002;16:89–99.
43. Sidhu JS, Farin FM, Omiecinski CJ. Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. Arch. Biochem. Biophys. 1993;301:103–113.
44. Sidhu JS, Liu F, Omiecinski CJ. Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: Requirement of dexamethasone and extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes. Exp. Cell Res. 2004;292:252–264.
45. Bader A, Zech K, Crome O, Christians U, Ringe B, Pichlmayr R, Sewing KF. Use of organotypical cultures of primary hepatocytes to analyse drug biotransformation in man and animals. Xenobiotica; The fate of foreign compounds in biological systems. 1994;24(7):623-33.
46. Kern A, Bader A, Pichlmayr R, Sewing KF. Drug metabolism in hepatocyte sandwich cultures of rats and humans. Biochemical pharmacology. 1997;54(7):761-72.
47. Langsch A, Giri S, Acikgoz A, Jasmund I, Frericks B, Bader A. Interspecies difference in liver-specific functions and biotransformation of testosterone of primary rat, porcine and human hepatocyte in an organotypical sandwich culture. Toxicology letters. 2009;188(3):173-9.
48. Bader A, Knop E, Kern A, Boker K, Fruhauf N, Crome O, Esselmann H, Pape C, Kempka G, Sewing KF. 3-D coculture of hepatic sinusoidal cells with primary hepatocytes-design of an organotypical model. Experimental cell research. 1996;226(1):223-33.
49. Dunn JC, Tompkins RG, Yarmush ML. Hepatocytes in collagen sandwich: Evidence for transcriptional and translational regulation. The Journal of cell biology. 1992;116(4):1043-53.

50. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. Biotechnology progress. 1991;7(3):237-45.
51. Bader A, Knop E, Böker KHW, Crome O, Frühauf N, Gonschior A, Christians U, Esselman H, Pichlmayr R, Sewing KF. Tacrolimus (FK 506) metabolism in primary rat hepatocytes depends on extracellular matrix geometry. NS Arch Pharm. 1996;353:461-473.
52. Acikgoz A, Karim N, Giri S, Schmidt-Heck W, Bader A. Two compartment model of diazepam biotransformation in an organotypical culture of primary human hepatocytes. Toxicology and applied pharmacology. 2009;234(2):179-91.

© 2014 Acikgöz et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=607&id=14&aid=5604