St. John’s wort formulations induce rat CYP3A23-3A1 independent of their hyperforin content

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List of nonstandard abbreviations: DMEM, Dulbecco’s modified Eagles medium; FCS, fetal calf serum; LBD, ligand binding domain; NEAA, non-essential amino acids; PCN, pregnenolone-16α-carbonitrile; NR, nuclear receptor; PXR, Pregnane X Receptor; SJW, St. John’s wort.
Abstract

The pregnane X receptor (PXR) is a ligand activated regulator of cytochrome P450 (CYP)3A enzymes. Among the ligands of human PXR is hyperforin, a constituent of St John’s wort (SJW) extracts and potent inducer of human CYP3A4. It was the aim of this study to compare the effect of hyperforin and SJW formulations controlled for its content on CYP3A23-3A1 in rats. Hyperiplant® was used as it contains a high hyperforin content and Rebalance® because it is controlled for a low hyperforin content. In silico analysis revealed a weak hyperforin-rPXR binding affinity, which was further supported in cell-based reporter gene assays showing no hyperforin-mediated reporter activation in presence of rPXR. However, cellular exposure to Hyperiplant® and Rebalance® transactivated the CYP3A-reporter 3.8 fold and 2.8 fold, respectively, and they induced Cyp3a23-3a1 mRNA expression in rat hepatoma cells compared to control 48-fold and 18-fold, respectively. In Wistar rats treated for 10 days with 400 mg/kg of Hyperiplant® we observed 1.8-times the Cyp3a23-3a1 mRNA expression, a 2.6-fold higher CYP3A23-3A1 protein amount and a 1.6-fold increase in activity compared to control. For Rebalance® we only observed a 1.8-fold hepatic increase of CYP3A23-3A1 protein compared to control animals. Even though there are differing effects on rCyp3a23-3a1/CYP3A23-3A1 in rat liver reflecting the hyperforin content of the SJW extracts, the modulation is most likely not linked to an interaction of hyperforin with rPXR.

Significance statement

Treatment with St John's wort (SJW) has been reported to affect CYP3A expression and activity in rats. Our comparative study further supports this finding but shows that the PXR-ligand hyperforin is not the driving force for changes in rat CYP3A23-3A1 expression and function in vivo and in vitro. Importantly, CYP3A induction mimics findings in humans but our results suggest that another so far unknown constituent of SJW is responsible for the expression and function modifying effects in rat liver.
Introduction

Nuclear receptors (NRs) function as transcriptional regulators and play a central role in maintaining homeostasis in living beings. Upon ligand-activation, NRs exert transcriptional activity on their target genes, adapt their expression level, and thus their activity to the present requirements including the state of health vs. disease, exposure to exogenous compounds, or developmental processes (Delfosse et al., 2015; Sladek, 2011). One NR of extraordinary relevance in drug metabolism is the xenosensor pregnane X receptor (PXR, NR1I2) which acts as a transcriptional modulator of a variety of genes involved in drug metabolism and transport (Sladek, 2011). One characteristic of PXR is its species-specificity (Jones et al., 2000). In detail, comparing PXR in humans and rodents in terms of target genes shows a broad overlap (Ihunnah et al., 2011), therefore a comparable function in the regulation of drug metabolism is expected in all species. The overlap in target genes is assumed to be explained by the highly conserved DNA binding domain of PXR, where the rodent and human PXR share a 96% sequence homology (Kliewer et al., 2002). Nevertheless, there are species-specific differences in the activating ligands of human and rodent PXR particularly of rat and mouse. Accordingly, it is challenging to translate findings in these preclinical species to the human system. The differences in activating ligands may be due to the low sequence similarity within the ligand-binding domain (LBD) of the protein, where the human LBD only shares 77 or 76% sequence similarity with the mouse or rat LBD, respectively (AbdulHameed et al., 2016; Ma et al., 2008). Known examples of human specific PXR ligands are rifampicin and hyperforin (Tirona et al., 2004).

Hyperforin is found in St. John’s wort (SJW) extracts which have been successfully used as herbal remedies in the treatment of mild to moderate depression (Friede et al., 2001). However, herbal remedies containing hyperforin bear the potential for drug-herb interactions. Indeed, in humans it is well established that co-administration of SJW with substrates of the cytochrome P450 enzyme CYP3A4 or the efflux transporter MDR1 (ABCB1, P-glycoprotein) can result in severely altered bioavailability of their substrates, as most impressively shown for cyclosporine and digoxin (Johne et al., 1999; Ruschitzka et al., 2000). Despite the \textit{in vitro} data on the lack of activation of rPXR by hyperforin (Tirona et al., 2004), there are reports showing an influence of SJW on known PXR target
genes in rodents. Ho et al. tested the impact of a 15-day SJW-treatment (150 or 300 mg/day) on the pharmacokinetics of indinavir, a known CYP3A and MDR1 substrate in rats. They observed a reduction in systemic indinavir exposure (AUC_{0-infinity} indinavir alone vs. indinavir plus SJW 150 mg/kg vs. indinavir plus SJW 300 mg/kg; 5.20 ± 1.09 vs. 0.76 ± 0.50 vs. 1.07 ± 0.26 µg/ml) which is most likely explained by a change in oral bioavailability as observed in an \textit{in situ} single pass intestinal perfusion model. Moreover, the rats treated with 150 mg/kg SJW exhibited an approximately twofold higher CYP3A activity as determined by measuring the N-demethylation of erythromycin in isolated liver microsomes (Ho et al., 2009). An induction of CYP3A-mediated 1-hydroxylation in liver microsomes isolated from rats treated with SJW (1000 mg/kg) was also observed by Qi et al. They tested the time-dependency of changes in midazolam pharmacokinetics in rats, and reported an about 2.6-fold increase in oral clearance of midazolam in rats treated with SJW for 7 days (Qi et al., 2005).

Importantly, SJW formulations differ in hyperforin content (Schäfer et al., 2019). Moreover, clinical data in humans show that SJW-associated interactions are clearly linked to the hyperforin content of the respective formulation (Arold et al., 2005). One low-hyperforin SJW extract approved in various countries for the short-term treatment of depression is Ze117. This formulation with the brand name Rebalance® exhibits pharmacological efficacy (Friede et al., 2001; Schrader, 2000), but no relevant impact on the pharmacokinetics of substrates of drug metabolizing enzymes and P-gp (Bosilkovska et al., 2016; Zahner et al., 2019). Considering the availability of the SJW formulations of differing hyperforin content, we sought to directly compare their \textit{in vitro} and \textit{in vivo} effect on the PXR-target gene CYP3A23-3A1 in rats. With this we intended to further contribute to the understanding of the inconsistent findings previously made \textit{in vitro} and \textit{in vivo} for rat vs. human PXR. To improve readability CYP3A23-3A1 (NCBI reference sequence NM_013105.2) will be named \textit{Cyp3a1} when describing mRNA data and CYP3A1 when reporting on protein expression throughout the manuscript.
Material and methods

Materials. The low-hyperforin containing Hypericum extract (Rebalance 250®, LOT200658 with 500 mg extract), was kindly provided by Zeller AG (Romanshorn, Switzerland). The high-hyperforin containing Hypericum extract (Hyperiplant Rx®, LOT1250620, 600 mg, Schwabe Pharma AG, Küssnacht, Switzerland) was commercially obtained. The formulated tablets (of Rebalance® and Hyperiplant®) were pulverized using the Mixer Mill MM400 (Retsch GmbH, Düsseldorf, Germany). If not otherwise stated all other chemicals including the pure hyperforin were obtained from Sigma Aldrich (Buchs, Switzerland).

Molecular modeling. The rat PXR primary sequence was obtained from the Uniprot server (ID: Q9R1A7) [https://www.uniprot.org/]. The sequence was used as input for the SWISS-MODEL protein modeling server to create a rat homology model [https://swissmodel.expasy.org/]. Homology model-based on the human orthologue template co-crystallized with hyperforin (ID: 1M13) was used for further analyses. The human crystal structure and the rat homology model were superimposed using backbone atoms. The hyperforin molecule from the human PXR binding site along with all resolved water molecules were copied to the rat model. The binding site residues of the rat model were optimized by selecting the best interacting rotamer from the library (H-bonding satisfied, no steric clashes if possible). Finally, both protein-ligand complexes were fully pre-processed using Protein Preparation Wizard in Schrodinger Maestro [Maestro, Schrödinger, LLC, New York, NY, 2020] assuming physiological pH of 7.4. Next, the interaction patterns in the hyperforin binding sites were compared. Finally, protein-ligand complexes were placed in the cubic periodic boundary system filled with TIP3P water molecules and MD simulations were run using Desmond software (version 2019-1 (Bowers et al., 2006)). For each species, five independent MD simulations were performed using different random number generator seeds. The OPLS_2005 force field was selected along with the time-step of the RESPA integrator set to 2 fs. After the default equilibration protocol, the production simulations with a duration of 4.8 ns were conducted in an NPT ensemble at 310 K regulated by a Nosé–Hoover thermostat with the atmospheric pressure maintained by a Martyna–Tobias–Klein barostat. The u-series algorithm was selected by default to treat long-range interactions,
while bonds to hydrogen atoms were treated with the M-SHAKE algorithm (Shaw et al., 2014). PDB formatted coordinate files for the human PXR-hyperforin complex (based on PDB entry ID 1M13) and the rat PXR-hyperforin homology model can be found in the supplementary data 9 and 10, respectively.

**Cell culture.** HepG2 (HB-8065; American Tissue Culture Collection ATCC, Manassas Virginia USA; RRID: CVCL_0027) were cultured in DMEM (with 4.5g/mL Glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate; Sigma-Aldrich) supplemented with 10% FCS (BioConcept Ltd, Allschwil, Switzerland) and 1% L-Glutamine (200 mM, BioConcept Ltd). H4IIE cells were kindly provided by Prof. Dr. Alex Odermatt (Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel). The rat hepatoma cells were cultured in DMEM (4.5g/mL Glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, Sigma Aldrich) supplemented with 10% FCS, 1% MEM NEAA solution (BioConcept Ltd), 1% L-Glutamine (200 mM, BioConcept Ltd), and 1% 1 M HEPES-Buffer (BiConcept Ltd). HepaRG cells were kindly provided by Dr. Jamal Bouitbir (Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel). These cells were cultured in William’s Medium E (1X, Thermo Fisher Scientific, without L-Glutamine), supplemented with 10% FCS, 1% L-Glutamine (200 mM, BioConcept Ltd), 5 µg/ml human insulin (Sigma Aldrich) and 50 µM hydrocortisone hemisuccinate (Sigma Aldrich), and 1% penicillin/ streptomycin (BioConcept Ltd). HepaRG cells were cultured for 14 days as progenitor cells prior to a 14-day differentiation into biliary cells and hepatocytes which was induced by addition of 2% DMSO (Guillouzo et al., 2007). All cells were kept at 37°C in a humidified atmosphere supplemented with 5% CO₂ and routinely monitored for possible mycoplasma contamination.

**Cell-based reporter gene assay performed in human and rat liver cells.** In the cell-based reporter gene assays we used the previously reported synthetic reporter gene construct XREM-CYP3A4-PREM-pGL3basic (Meyer zu Schwabedissen et al., 2008), the commercially available pRL-TK (Promega, Dübendorf, Switzerland), and pEF6-based (invitrogen) expression plasmids encoding for human (Ferreira et al., 2019) or rat PXR. The latter was cloned from rat liver cDNA using the primer pair: rPXR-cds-for 5′-CAGTCCAGCAGACACAGATGTAACCTG -3′ and rPXR-cds-rev 5′-
CTGCTCCGTGAGATCTCCACTCAG-3. The resulting 1361bp-amplicon was ligated into pEF6/V5-His TOPO (invitrogen). After amplification of the plasmid in E. coli the insert was controlled by Sanger Sequencing (Microsynth, Balgach, Switzerland). For the cell-based reporter gene assay, 50 000 HepG2 cells were seeded in 24 well plates. One day after seeding, the cells in one well were transfected with 250 ng of the respective expression plasmid, 250 ng of the reporter gene construct, and 25 ng of the pRL-TK. 2 µl jetPRIME® (Chemie Brunschwig, Basel, Switzerland)/1 µg of DNA were used as transfection reagent. Medium was changed 4 hours after transfection. On the next day, the transfected cells were treated with hyperforin (1 µM), pregnenolone 16α-carbonitrile (PCN, 10 µM), rifampicin (10 µM), Rebalance® (2.5 mg/ml), or Hyperiplant® (4.5 mg/ml). The latter were prepared as a stock solution, where 1% of the respective milled tablets was dissolved in 2 ml DMSO prior to addition to the culture medium (1/100) (Schäfer et al., 2019). Concentrations of the formulations in the cell based experiments were calculated as previously described (Schäfer et al., 2019) considering the currently clinically used antidepressant treatment regimens. Treatment was performed in the dark to avoid phototoxicity. After 24 hours, cells were lysed and activities of the Firefly and the Renilla luciferase were measured using the Dual-Luciferase Assay System (Promega) and the plate reader Infinite M200 Pro (Tecan, Männedorf, Switzerland) according to the manufacturer’s instruction.

Animal study. All animal procedures were approved by the cantonal veterinary authority Basel-Stadt, Switzerland (License number 3092). Male Wistar rats obtained from Janvier-Labs (Le Genest-Saint-Isle, France) were housed in group cages, at a 12-hour light cycle and 24°C with water and food ad libitum. Experiments were performed following the ARRIVE 2.0 guideline for care and use of laboratory animals (Percie du Sert et al., 2020), and the principles of the CRUS Policy for Animal Research by swissuniversities (CRUS 2013). Animal group sizes were first estimated using the resource equation approach with a degree of freedom between 10 (minimum) and 20 (maximum) for the error term in an analysis of variance (ANOVA) (Arifin and Zahiruddin, 2017) which resulted in an expected number of 5 - 7 animals per group (Hyperiplant®, Rebalance®, or control). Six animals per group were selected, as this group size was expected to allow us to detect differences of around 35 -
55% assuming a standard deviation of 27.5% with the type 1 error (\(p\)) at the level of 5% (\(p = 0.05\)) with a power of the study at 80%. The treatments were prepared by suspending the respective amount of the SJW powder in the suspension mixture consisting of 0.5% methylcellulose and 0.1% Tween 80 in water. Suspensions were stirred overnight and kept in the dark to avoid degradation of light sensitive ingredients. Before the start of the experiment, rats aged 7 weeks were randomly assigned to the different treatment groups (Hyperiplant®, Rebalance®, or control). Blinding was not possible during the in vivo phase of the experiment. Rats received 400 mg/kg body weight of the respective SJW formulation in a volume of 2.8 ml/kg body weight by oral gavage. This dosage was shown to have an antidepressant effect in rats and to induce genes involved in drug metabolism (Rezvani et al., 1999; Shibayama et al., 2004). The suspended formulations or the suspension mixture alone (control) were orally administered with a feeding tube (Instech Laboratories, Leipzig, Germany) on 10 consecutive days between 9 and 10 am. On day 11, the rats were sacrificed by CO\(_2\) with subsequent organ harvest. Tissue samples were snap-frozen in liquid nitrogen before storage at -80 °C until further use.

**Gene expression analysis of PXR target genes.** H4IIE and HepaRG cells were seeded in 6 well plates (Sarstedt, Sevelen, Switzerland) at a density of 300,000 cells per well. H4IIE cells were cultured for 24 hours before treatment was started, while HepaRG cells were differentiated as described above. For the treatment, hyperforin, PCN, and rifampicin were used at a concentration of 1, 1, and 20 μM, respectively. The SJW formulations Rebalance® (2.5 mg/ml or 4.5 mg/ml for H4IIE, and 0.25 mg/ml for HepaRG cells), or Hyperiplant® (4.5 mg/ml for H4IIE, 0.45 mg/ml for HepaRG cells) were prepared as described above. Due to the high sensitivity of HepaRG cells which exhibited a pronounced reduction in viability upon exposure to the formulation suspensions, a ten times lower concentration was used for the herein reported treatment. H4IIE cells were treated for 48 hours and HepaRG cells were treated for 72 hours with a change of treatment medium every day. Frozen liver tissue pieces were milled using the Mixer Mill MM400. Extraction of mRNA was performed using TRI Reagent® (1 ml/100 mg tissue or 1 ml/well; Sigma-Aldrich) according to the manufacturer’s instructions. Reverse transcription was performed with 1000 ng of RNA using the High-Capacity
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cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Reinach, Switzerland). The mRNA expression of rCyp3a1 (5’-TCTGTGCAGAAGCATCGAGT-’3 and 5’-GGCTGTGATCTCATATCG-’3), CYP3A4 (5’-ATCATTTGCTGTCTCAACCTTCACTAC-’3 and 5’-TGCTTCCCAGATTTCTCT-’3) was measured with the SYBR®-Green RT-PCR-Kit (Thermo Fisher Scientific). β-Actin (rat: 5’-GGAGATTACTGCCCTGGCTCCTA-’3 and 5’-GACTCATCGTACTCCTGCTTGCTG-’3; human: 5’-CCAACCGCGGAAGATGA-’3 and 5’-CCAGAGGCGGTACAGGGGATAG-’3) was used as housekeeping gene. The analysis was performed with the QuantStudio® 5 (Applied Biosystems, Thermo Fisher Scientific) using the QuantStudio® Design & Analysis Software 1.5.1. Gene expression was evaluated using the 2^ΔΔCT method described by Livak & Schmittgen (Livak and Schmittgen, 2001). The Ct-value of the gene of interest was normalized to the mean of β-Actin and the results are presented as a fold of study mean.

Isolation of liver microsomes. Rat liver microsomes of 5 animals per treatment were isolated as previously described (Haduch et al., 2018; Hiroi et al., 1998). Briefly, 1 g of liver was rinsed with Tris/KCl (20 mM/0.15 M) buffer before homogenization with the Homogenizer Potter S (Sartorius, Göttingen, Germany). The homogenate was centrifuged for 20 minutes at 10,000 x g and 4 °C before centrifugation of the supernatant at 100,000 x g and 4°C for 1 hour. The pellet was suspended in Tris/KCl (20 mM/0.15 M) buffer, and homogenized using the Polytron PT1200E (Kinematica AG, Malters, Switzerland). After an additional centrifugation at 100,000 x g and 4°C for 1 hour the pellet was suspended in the Tris/Sucrose buffer (20 mM/0.25 M) using the Polytron PT1200E prior to storage at −80 °C until further experiments. Protein content was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and the Microplate Reader Infinite M2000 Pro (Tecan).

Western blot analysis. For protein expression analysis, 20-40 mg of each liver tissue were suspended in subcellular fractionation buffer (SF, 250 mM sucrose, 20 mM Hapes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA) containing the proteinase inhibitors phenylmethylsulfonylfluorid 1 mM, leupeptin 5 μg/ml and aprotinin 2 μg/ml before homogenization using the Dounce tissue
grinder from Wheaton (Millville, USA). After centrifugation at 720 x g and 4°C for 5 minutes, 
the supernatant was centrifuged at 10,000 x g and 4°C for 10 minutes. The pellet containing the 
liver protein lysate was resuspended in lysis buffer (50 mM Tris HCl, 150 mM NaCl, 0.5% 
sodium deoxycholate, 0.1% SDS, 1% Triton X-100) followed by quantification of protein content 
as described above. Prior to SDS 10%-polyacrylamide gel electrophoresis using the Mini-PROTEAN 
Tetra™ cell system (Bio-Rad Laboratories AG, Cressier, Switzerland), 10 μg of liver protein lysate 
supplemented with 4 x Laemmlli-solution were incubated at 37°C and 300 rpm for 60 minutes. After 
the separation the proteins were transferred onto a nitrocellulose membrane using the Blot®L1 system 
(GenScript, Piscataway, U.S.A.). After an one hour incubation with 5% non-fat dry milk (Blotto, Carl 
Roth GmbH, Karlsruhe, Germany) in TBS-T (NaCl 140 mM, KCl 2.5 mM, Tris-Ultra 25 mM 0.1% 
Tween 20) at room temperature, the membranes were exposed to the respective primary antibody 
solution (antibody diluted in 1% BSA-TBS-T) overnight at 4°C (anti-CYP3A1, ab1253, 1:10,000; 
Merck Millipore, Darmstadt, Germany, RRID: AB_90531, or anti-calnexin, ADI-SPA-865, 1:1,000, 
Enzo Life Sciences, Lausen, Switzerland, RRID: AB_10618434). Thereafter the membranes were 
washed several times with TBS-T, followed by incubation with the secondary antibody at room 
temperature for 1 hour (HRP-labeled anti-rabbit, 1:2,000, Cell Signaling Technology, Danvers, USA, 
RRID: AB_2099233). Immobilization of the secondary antibody was visualized using the 
ChemiDoc™ XRS imaging system equipped with the Image Lab software (Version 6.0) and a 
1:1-mixture of the Clarity Western ECL Substrate Peroxide solution and the Luminol/enhancer 
solution (Bio-Rad Laboratories, Cressier, Switzerland). Band intensity was densitometrically 
analyzed.

**Immunohistochemical staining.** Tissue slides were deparaffinized using Ottix plus (DiaPath, 
Martinengo, Italy) before rehydration in a decreasing ethanol series. Heat induced epitope retrieval 
was performed with the citrate buffer (BioSB Inc., Santa Barbara, USA, pH 6.1) in the TintoRetriever 
pressure cooker. After a two-hour incubation with TBS-0.025% Triton X 100 (TBS-T) containing 5% 
donkey serum and 1% bovine serum albumin (BSA), the anti-CYP3A1 antibody (ab1253, Merck 
Millipore diluted 1:500, in 1% BSA/TBS) was added to the slides and incubated at 4°C overnight in a
humidified atmosphere. After several washings in TBS-T, the tissue sections were exposed to 3% H$_2$O$_2$ for 15 minutes before an additional wash and an incubation with the HRP-labeled secondary antibody (A16035, Life Technologies diluted 1:200 in 1% BSA/TBS-T, RRID: AB_2534709) for 2 hours. After washings with TBS-T the tissue sections were incubated in 50 mM Tris-buffer (pH 7.6) at 37°C before adding the DAB-staining solution consisting of 10 mg/ml 3,3-diaminobenzidine in 50 mM Tris-buffer supplemented with 0.02% H$_2$O$_2$. Nuclei were stained with Hemalum solution acid acc. to Mayer (Carl Roth) before dehydration in an increasing ethanol series. After fixation with Roti®-Seal (Carl Roth) the Leica DMi8 Microscope (Carl Zeiss, Microscopy, Munich, Germany) was used for image acquisition. Images were taken by an experimenter who was blinded to group allocation.

Quantification of hyperforin in Hyperiplant$^\text{®}$ and Rebalance$^\text{®}$. 100 mg of the pulverized tablet were suspended in 0.5% methylcellulose and 0.1% Tween 80 in water and stirred overnight. On the next day, suspensions were centrifuged at 13,000 rpm for 5 minutes. Supernatants were filtered through a disposable syringe filter (Micropur PTFE, pore-size: 0.45 μm, Altmann Analytik, Munich, Germany) of which 100 μl were combined with 900 μl DMSO. 10 μl of these solutions were injected for quantification of hyperforin using high-performance liquid chromatography with UV detection (HPLC-UV). Conditions for HPLC analysis were the same as previously reported (Schäfer et al., 2019). The sum of hyperforin and adhyperforin was considered when calculating the hyperforin content. For both hyperforin and adhyperforin a calibration curve was prepared ranging from 1.0 – 0.007 mg/ml. Analysis was performed on an Alliance 2690 chromatographic system coupled to a PDA996 detector (Waters, Milford, MA). The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% trifluoroacetic acid. Hyperforin was separated using a 115 Zorbax Eclipse XDB-C8 Narrow-Bore column (2.1 × 150 mm, 3.5 μm; Agilent, Santa Clara, CA) with a gradient of 50%–100% B in 20 minutes, then 100% B for 15 minutes, followed by 50% B for 10 minutes at a flow rate of 0.4 ml/minute. Separations were performed at 30 °C and detection was at 272 nm. During preparation, the samples were kept in the dark to avoid degradation of light sensitive ingredients.


In vitro assessment of CYP3A activity in liver microsomes. The activity of the CYP enzymes was determined by measuring the rate of 2β- and 6β-hydroxylation of testosterone in rat liver microsomes. For in vitro activity measurement an incubation mixture containing 50 µl of the microsomal suspension (1 mg protein/ml), 30 µl phosphate buffer (50 mM Na2HPO4 adjusted to pH 7.4 with 50 mM KH2PO4), and 900 µl incubation buffer consisting of phosphate buffer supplemented with MgCl2 (3.0 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), and glucose-6-dehydrogenase phosphate (1.7 U/ml) prepared. The reaction began after adding 20 µL testosterone (100 µM) and placing the samples in a water bath at 37°C. The reaction was stopped after 15 minutes of incubation by adding 200 µl of methanol and transferring the samples on ice. Testosterone and its metabolites 2β- and 6β-hydroxytestosterone were extracted from the incubation mixture to chloroform (6 ml). After evaporation under nitrogen and drying, the residue was dissolved in 100 µl methanol. 10 µl of the resulting solution was loaded onto the C18 SunFire (Waters) column (3.5 µm, 3.0 x 150 mm) with a precolumn (VanGuard from Waters). The mobile phase consisted of methanol-water-acetonitrile (39:60:1) (A) and methanol-water-acetonitrile (80:18:2) (B) with a gradient of 0% to 100% B in 13 min and 100% B from 13-15 min. The gradient started after 2 min at a flow rate of 0.3 ml/min. The column temperature was 40 °C. Absorbance of testosterone and its metabolites was measured at a wavelength of 254 nm. Retention times for the reaction products were 8.6, 10.4, and 12.2 minutes for 6β-hydroxytestosterone, 2β-hydroxytestosterone, and testosterone, respectively. For calculating the absolute values of metabolites formed, the external standard method was applied, according to Sonderfan et al. (Sonderfan et al., 1987). Briefly, five mixtures containing increasing concentrations of 6β-hydroxytestosterone and 2β-hydroxytestosterone were used (0.01, 0.02, 0.05, 0.1, 0.2 mM). The standard containing mixtures were treated the same way as the samples, however, they were not incubated at 37°C.

Statistical analysis. The herein reported data sets were analyzed using Microsoft Excel (Microsoft, Redmond, USA) and the GraphPad Prism software 9.3.1 (GraphPad Software, San Diego, USA). All error bars are represented as a mean ± standard deviation (SD). Tests for statistical analysis are described in the context of data presentation. A p-value below 0.05 was considered statistically
significant. We report an exploratory data analysis. Consequently, calculated $p$-values should be interpreted descriptive.
Results

Hyperforin exhibits different binding modalities for the rat or the human PXR. At first we analyzed in silico the binding of hyperforin to the ligand binding domain (LBD) of human or rat PXR. The structure of the LBD of rat PXR was based on a homology model and we focused on residues with immediate contacts to the ligand (within 5 Å distance). In accordance with previous findings (Ngan et al., 2009; Watkins et al., 2003) hyperforin finds a well suited binding pocket within the human PXR. However, in the rat PXR there are differences in amino acid residues of which some severely decrease the binding affinity of hyperforin to the rat PXR. As shown in Figure 1 the rat PXR contains an Isoleucine (ILE144) instead of the Glutamine (GLN285) in the human PXR (N.B. X-ray structure residue numbering is used for the human structure, but a full sequence numbering is used for the rat homology model). The GLN285 allows a strong and stable binding to the hyperforin by means of an H-bond, which is not present in the rat PXR. Instead, ILE144 in rat PXR forms compensating hydrophobic interactions, but also leaves nearby Histidine (HIS186) without an interaction partner. Next, the Glutamine (GLN266) in the rat PXR – due to its larger size and flexibility – cannot form an analogous stable H-bonding interaction like the Histidine HIS407 in the human PXR to one of the carbonyl groups of hyperforin. In rat PXR, the H-bond is lost in four of five MD simulations, whereas in human PXR it can be maintained in four of five simulations (see Supplementary Figure 1A and B). These simulations confirm the low relative stability of the rat PXR-hyperforin complex and the favorable interaction in the human PXR-hyperforin complex. Differences in case of lipophilic amino acid residues (rMET68 vs. hLEU209; rILE70 vs. hVAL211; rLEU102 vs. hMET243; rVAL105 vs. hMET246; rLEU182 vs. hMET323) do not seem to affect the binding mode, as they still preserve the lipophilic character of the hyperforin-receptor interaction. Even if positioned at the outer layer of the binding pocket the change of the amino acid residue from Leucine LEU206 in human PXR to Serine SER65 in rat PXR further reduces the binding affinity as a hydrophobic interaction is preferred to a polar one. Overall, despite the fact that hyperforin can be sterically accommodated in the binding site of the rat PXR ligand binding domain, the interaction pattern – especially with regard to H-bonding interactions – is much weaker when compared to the human PXR.
Cell-based reporter gene assays support that hyperforin is not involved in rat PXR-mediated transactivation of the CYP3A4-reporter. Despite the in silico finding suggesting low interaction of hyperforin with the rat PXR, we sought to assess the impact of hyperforin and the two SJW formulations in cell-based reporter gene assays monitoring the activation of the synthetic XREM-CYP3A4-PREM-promoter construct in cells co-transfected with a plasmid encoding for the Renilla luciferase. We determined transactivation of the CYP3A4-reporter (Firefly luciferase) in HepG2 cells either transfected with the human PXR (Figure 2A) or the rat PXR (Figure 2B). In cells expressing human PXR, rifampicin, hyperforin, and Hyperiplant® led to a 17.18, 44.46, and 35.77-fold increase in the normalized luciferase activity, respectively. No activation was observed in Rebalance® treated cells. In HepG2 cells expressing rat PXR, we observed much higher basal activity of the CYP3A4-reporter compared to cells transfected with human PXR. Regardless of this observation, we detected a 4.4-fold, a 3.8-fold, and a 2.8-fold induction of the reporter activity after treatment with PCN, Hyperiplant, and Rebalance. Exposure to hyperforin did not affect the reporter activity.

No impact of hyperforin on endogenous rCyp3a1 expression in rat hepatoma cells. In a next step we assessed the impact of hyperforin and the SJW formulations on the rCyp3a1 mRNA expression in rat hepatoma cells. Differentiated HepaRG cells served as a model for human hepatocytes. In HepaRG cells we observed a 3.4-fold induction of the CYP3A4 mRNA expression after exposure to hyperforin and a 5.9-fold induction after exposure to Hyperiplant®, while no change was observed after a 72-hour exposure to Rebalance® (Figure 3A). In accordance with our findings in the cell-based reporter gene assay we observed induction of the rCyp3a1 mRNA by about 5- and 2-fold in H4IIE cells treated with Hyperiplant® and Rebalance®, respectively, while hyperforin did not exert an effect on rCyp3a1 mRNA in these cells (Figure 3B). Inducibility was controlled with PCN (rCyp3a1 mRNA expression as fold of study mean ± SD; 27.68 ± 5.16) and rifampicin (CYP3A4 mRNA expression as fold of study mean ± SD; 3.68 ± 0.19) for the rat and the human system, respectively (Figures 3C and D). As shown in Supplementary Figure 2, we also observed differences in the inductive efficacy of Hyperiplant® and Rebalance® in H4IIE cells after exposure to the same concentration of the
formulations. Supplementary Figures 3A and 3B show quantitative expression levels of b-Actin (raw Ct values) measured in H4IIE and HepaRG cells, respectively.

**Long-term oral treatment of rats with Hyperiplant® or Rebalance® increases CYP3A1 expression.** Observing induction of rCyp3a1 in rat hepatoma cells by both Hyperiplant® and Rebalance®, we next sought to investigate the influence of the two SJW formulations on the hepatic rCyp3a1 mRNA expression and CYP3A1 abundance in rats. To ensure induction we treated rats orally on 10 consecutive days with formulations characterized for their hyperforin content (Supplementary Figures 4 and 5), and probed their livers for rCyp3a1/CYP3A1. Immunohistochemical staining confirmed the presence of CYP3A1 in the livers of the differently treated animals as shown in Figure 4A, with no obvious change in distribution. Assessing the amount of rCyp3a1 mRNA we observed a 1.8-fold increased transcript level in rats treated with Hyperiplant®. However, no change was detected for Rebalance® compared to control animals (Figure 4B). Moreover, no change in mRNA expression was observed for rCyp3a2, which is also considered an orthologue of CYP3A4. Supplementary Figure 3C shows quantitative expression levels of b-Actin (raw Ct values) measured in the liver tissue of the treated animals. Finally, we assessed the amount of CYP3A1 by Western blot analysis of rat liver lysates (representative image in Figure 4C). As shown in Figure 4D, densitometric analysis revealed that animals treated with either Hyperiplant® (mean CYP3A1 protein expression as fold of solvent control; 2.649 ± 0.351; n = 5) or Rebalance® (1.830 ± 0.2557; n = 5) showed a higher amount of CYP3A1 in liver compared to control rats (1.000 ± 0.207; n = 6 animals). Western blots used for densitometric analysis are summarized in Supplementary Figure 6.

Hyperiplant® increased CYP3A activity in microsomes isolated from rat livers. Activity of CYP3A was measured in microsomes isolated from livers of the treated rats by determining the *in vitro* formation of the testosterone metabolites 6β- or 2β-OH-testosterone. Formation of 6β-OH-testosterone was 547.8 ± 171.3, 852.6 ± 197.7, or 548.4 ± 180.8 nmol mg protein⁻¹ min⁻¹ in liver microsomes isolated from animals treated for 10 days with solvent control, Hyperiplant® or Rebalance® (n = 5 animals; mean ± SD, Figure 5A), respectively. A statistically significant increase
was observed in *in vitro* 6β-OH-testosterone formation in liver microsomes isolated from animals treated with Hyperiplant® (1.6-fold increase) compared to those isolated from rats treated with Rebalance®. Analyzing the data as % of solvent control revealed a mean ± SD of 155.7 ± 36.09% for Hyperiplant® compared to Rebalance® (100.1 ± 20.57%), or solvent control (100.0 ± 31.27%). As shown in **Figure 5B**, no such effect was observed for the formation of 2β-OH-testosterone. We observed a formation rate of 1911 ± 762.8, 2189 ± 613.2, and 1782 ±775.1 nmol mg protein⁻¹ min⁻¹ 2β-OH-testosterone in liver microsomes isolated from animals treated for 10 days with solvent control, Hyperiplant® or Rebalance® (each n = 5 animals; mean ± SD), respectively. The chromatograms of standard testosterone metabolites and of testosterone metabolites in microsomes of differently treated animals can be found in **Supplementary Figures 7** and **8**, respectively.
Discussion

In this study we report on the direct comparison of two St John's wort formulations and their impact on the expression of the metabolizing enzyme CYP3A1 in rats. We compared the formulation Hyperiplant® which contains a high amount of hyperforin (3-6 mg/100 mg dry extract) and Rebalance®. Importantly, the latter formulation contains only a low amount of hyperforin (0.2 mg/100 mg dry extract) and has been verified for the lack of hyperforin-mediated interactions in a clinical study in humans (Zahner et al., 2019). Here, Rebalance® (aka Ze117) neither affected activity of drug metabolizing enzymes, nor P-glycoprotein as determined using a probe drug cocktail (Zahner et al., 2019). The clinical assumption of Rebalance® not affecting the hPXR-mediated transactivation of CYP3A4 is in line with the in vitro findings of our study, where we compared the impact of Hyperiplant® and Rebalance® on the transactivation of the CYP3A4-promoter in cell-based reporter gene assays (Figure 2A), or the effect on the CYP3A4 mRNA expression in HepaRG cells (Figure 3A). However, comparing the two formulations for their impact on rat PXR-mediated transactivation and on the rCyp3a1 mRNA expression in the rat hepatoma H4IIE cells, revealed a rather unexpected result. Indeed, we observed for both Rebalance® and Hyperiplant® increased transactivation of the promoter (Figure 2B) and enhanced expression of the rPXR target gene (Figure 3B). However, in line with our in silico findings and previous reports on rPXR and hyperforin (Tirona et al., 2004), there was no effect of this constituent of St. John’s wort on the experimental endpoints we measured for rPXR in vitro. Our in silico analysis involving molecular modeling of the ligand binding domain (LBD) confirmed the good embedding of hyperforin in the large and flexible binding pocket of human PXR. In contrast to the rat PXR there are discrepancies in some amino acid residues within the LBD that lead to a considerable loss in binding affinity. This statement mainly refers to the amino acids GLN285, HIS407, MET243, and LEU206 in the human PXR and ILE144, GLN266, LEU102, and SER65 in the LBD of rat PXR. The amino acids HIS407 and LEU206 in the LBD of human PXR are also of importance for the binding affinity of the cholesterol lowering drug SR12813. Indeed, Watkins et al. compared the human and mouse orthologue of PXR identifying the amino acids SER208 (P205 in mouse), HIS407 (Q404 in mouse) and ARG410 (GLN407 in mouse) defining the species different
SR12813 binding affinity. Their *in silico* finding was further validated experimentally showing that humanized mouse PXR gained activation by SR12813, while transaction by PCN was lost (Watkins et al., 2001). Accordingly, slight amino acid variations modify the binding of ligands to PXR (Heuvel, 2020), thereby contributing to the observed species differences in the spectrum of activating ligands.

Nevertheless, there are multiple studies reporting increased function of PXR-target genes in rats treated with St. John’s wort (Ho et al., 2009; Qi et al., 2005). For *rCyp3a1* mRNA expression Shibayama et al. specifically report an about 3.5-fold increase in rats treated with 400 mg/kg for 10 consecutive days (Shibayama et al., 2004). In our study we compared two formulations. For a 10-day treatment with 400 mg Hyperiplant® per day we observed increase of not only the mRNA level of *rCyp3a1*, but also the CYP3A1 protein amount in liver, which translates into higher enzyme activity as measured determining the formation of 6β-hydroxytestosterone in liver microsomes. No impact was observed for the formation of 2β-hydroxycholesterol. Notably, CYPs of the 3A family are highly involved in the 6β-hydroxytestosterone formation while they weakly catalyze 2β-hydroxycholesterol formation (Waxman et al., 1991; Yamazaki and Shimada, 1997). For the treatment with 400 mg/kg Rebalance® per day we observed higher levels of CYP3A1 protein in the livers, but no impact on *rCyp3a1* mRNA or the enzyme activity. Taken together, our findings on Hyperiplant® and Rebalance® indicate a mimicking effect on a PXR-regulated drug-metabolizing enzyme, hence comparable to the expected effect of SJW formulations differing in hyperforin content in humans. Nonetheless, compared to a remarkable influence in humans as for example reported by Markowitz et al. showing a 50% AUC decrease and a 2-fold clearance increase of the CYP3A4 substrate alprazolam after St. John’s wort treatment (Markowitz et al., 2003), results of our *in vivo* study are rather discreet. Furthermore, our *in silico* and *in vitro* findings on hyperforin clearly support that this constituent is not driving the observed *in vivo* changes in rats. However, the *in vitro* data indicated a difference in efficacy of Hyperiplant® and Rebalance® in H4IIE cells. The difference in effects may be linked to other constituents of the extracts. Formulated SJW extracts are, according to the pharmacopeia, normalized to the naphthodianthrones calculated as total hypericins. No information is provided for other constituents including flavonol derivatives, biflavones, and proanthocyanidin present in the
formulations (Nahrstedt and Butterweck, 1997). However, the manufacturers of Hyperiplant® and Rebalance® are reporting both the hypericin and the hyperforin content of their product (Hyperiplant®, Schwabe Pharma AG, Rebalance®, Zeller AG). Despite those constituents, the composition of Rebalance® and Hyperiplant® may greatly vary even if they are extracts of the same plant material, due to the fact that the two extracts are generated by applying different extraction methods (Nicolussi et al., 2020). Indeed, for Hyperiplant® the extractant is methanol while ethanol is used for St. John’s wort extraction in Rebalance®. The impact of different extraction methods on the composition of extracts from St. John’s wort has nicely been shown by Avato and Guglielmi. They report not only differences in hypericin and hyperforin content but also for other components (Avato and Guglielmi, 2004). Some of these components belong to the class of flavonoids including rutoside or quercetin for which interaction with nuclear receptors is assumed (Avior et al., 2013). In this context it seems noteworthy, that early data from Moore et al., who tested several of these flavonoids for human PXR-mediated transactivation rather suggested that those molecules are not the driving force of hPXR-mediated interactions (Moore et al., 2000). Nevertheless, for rutoside a two-fold increase of the reporter gene was observed in presence of human PXR (Moore et al., 2000). Furthermore, data from Lau et al. suggest binding at least of quercetin to human PXR, and transactivation of CYP3A4 at high concentrations, but the group did not see a similar effect when testing quercetin for the rPXR-mediated transactivation (Lau and Chang, 2015). The impact of quercetin on human PXR is further supported by clinical findings in which a 13 day treatment of 18 healthy male volunteers reduced the systemic exposure of the CYP3A substrate midazolam (Duan et al., 2012).

Even though we are not able to report on the constituent and therefore the mechanism underlying the in vitro activation of rPXR or in vivo induction of rat CYP3A1, we conclude that hyperforin is indeed not the driving constituent for the changes in rCyp3a1/CYP3A1 expression and function in vivo. This differs from the situation in humans, where hyperforin activates PXR and induces PXR target genes (e.g. CYP3A4). This effect depends on the concentration of hyperforin. Consequently, the formulation-specific effect in humans is due to the changes in hyperforin content of the different commercial St. John’s wort formulations. Nevertheless, our data support that St. John’s wort extracts
exert an inducing effect in rat liver but to a lower extent than expected from the findings in humans and most likely by a hyperforin-independent mechanism.
Data availability statement

The authors declare that all processed data supporting the findings of this study are available within the paper and its Supplemental Data. Raw data are available on request from the corresponding author.

Authorship contributions

Participated in research design: Schäfer and Meyer zu Schwabedissen

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Wrote or contributed to the writing of the manuscript: Schäfer, Rysz, Schädeli, Hübscher, Khosravi, Fehr, Potterat, Smieško, and Meyer zu Schwabedissen
References


Heuvel JV (2020) Species differences in pregnane X receptor (PXR) activation: Examination of common laboratory animal species.


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Figure legends

Figure 1. Interaction between human (red) and rat (orange) PXR amino acid residues and hyperforin (green). A rat homology model was created with the crystallized human PXR-hyperforin structure (ID: 1M13) using the SWISS-MODEL protein modeling server. Comparison and analysis of rat and human amino acid residues in the PXR binding pocket and their positions were fully pre-processed using Protein Preparation Wizard in Schrodinger Maestro. (A) human Glutamine (GLN) 285 vs. rat Isoleucine (ILE) 144; (B) human Histidine (HIS) 407 vs. rat Glutamine (GLN) 266; (C) human Leucine (LEU) 206 vs. rat Serine (SER) 65.

Figure 2. Cell-based reporter gene assay testing the transactivation of CYP3A4 in hepatoma cells of human origin. HepG2 cells were transfected with the synthetic reporter construct XREM-CYP3A4-PREM and either the human (A) or the rat (B) PXR. After 24 hours cells were treated with hyperforin (1 μM), Hyperiplant® (4.5 mg/ml), Rebalance® (2.5 mg/ml), or a prototypical inducer. For human PXR (hPXR) we used rifampicin (10 μM), and for rPXR pregnenolone-16α-carbonitrile (PCN; 10 μM). Luminescence was measured 48 hours after treatment was initiated. Data are reported as the ratio of luminescence of the Firefly luciferase normalized to that of the Renilla luciferase shown as mean ± SD. Experiments were performed three times. Each data point represents the mean of three replicates in one independent measurement. * p ≤ 0.05, one-way ANOVA corrected for multiple comparisons (Dunnett’s test).

Figure 3. Impact of hyperforin, Hyperiplant® or Rebalance® on the CYP3A-transcription in cells of human or rat origin. (A) Differentiated HepaRG cells were treated with hyperforin (1 μM), Hyperiplant® (0.45 mg/ml) or Rebalance® (0.25 mg/ml) for 72 hours. The amount of CYP3A4 mRNA was measured by quantitative real-time PCR. (B) H4IIE cells were exposed to hyperforin (1 μM), Hyperiplant® (4.5 mg/ml) or Rebalance® (2.5 mg/ml) for 48 hours prior to mRNA extraction and quantification of rCyp3a1. Rifampicin served as inducibility control for HepaRG cells (C) and PCN for H4IIE cells (D). Data are shown as the mean fold of study mean ± SD. Experiments were performed three times. Each data point represents the mean of three replicates in one independent
measurement. * $p \leq 0.05$, one-way ANOVA corrected for multiple comparisons (Dunnett’s test) (A, B); unpaired t-test (C, D); PCN, pregnenolone-16α-carbonitrile.

**Figure 4. Hepatic Cyp3a1/ CYP3A1 expression in rats treated with Hyperiplant® or Rebalance®.**
Rats were orally treated for 10 consecutive days with 400 mg/kg of the respective formulation or the suspension mixture alone as control. (A) Immunohistochemical staining of livers of differently treated animals. (B) The mRNA expression of rCyp3a1 was detected by real-time PCR. Data are reported as fold of solvent control. Indicated is the mean ± SD of n = 5-6 animals, each value was measured in duplicates. (C) Representative Western blot of 3 randomly selected control-, Hyperiplant®- and Rebalance®-treated animals with calnexin used as loading control. (D) Densitometric analysis of CYP3A1 protein expression in rat liver microsomes obtained by Western blot analysis. * $p \leq 0.05$, Kruskal Wallis Test followed by Dunn’s multiple comparisons was used for n = 5-6 animals in mRNA expression analysis. Densitometric testing of n = 5-6 animals was analyzed with one-way ANOVA followed by Dunnett’s multiple comparisons test, both calnexin lines were taken into consideration for the calculation.

**Figure 5. rCYP3A activity in microsomes isolated from livers of animals after 10 days of treatment with Hyperiplant® or Rebalance®.** CYP-activity was determined by measuring the *in vitro* formation of 6β-OH-testosterone (A) and 2β-OH-testosterone (B) in liver microsomes isolated from rats. Livers were harvested from rats treated on 10 consecutive days with solvent control, Hyperiplant® (400 mg/kg) or Rebalance® (400 mg/kg). Data are shown as mean ± SD of n = 5 animals. Each data point is the mean of three independent measurements. * $p \leq 0.05$; one-way ANOVA corrected for multiple testing (Dunnett’s test).
Figure 1
Figure 2

(A) HepG2 + hPXR

(B) HepG2 + rPXR
Figure 3

A

HepaRG

CYP3A4 mRNA expression

[DMSO, Hyperforin, Hyperplant, Rebalance]

B

H4IIE

Cyp3a1 mRNA expression

[DMSO, Hyperforin, Hyperplant, Rebalance]

C

HepaRG

CYP3A4 mRNA expression

[DMSO, Rifampicin]

D

H4IIE

Cyp3a1 mRNA expression

[DMSO, PCN]
Figure 4

A

control

Hyperiplant

Rebalance

B

rCyp3a1 mRNA expression [fold of control]

count

Hyperiplant Rebalance

rCyp3a2 mRNA expression [fold of control]

control Hyperiplant Rebalance

C

1 2 3 1 2 3 1 2 3

58 kDa 48 kDa

116 kDa 90 kDa

rCYP3A1

calnexin

D

rCYP3A1 protein expression (fold of solvent control)

solvent Hyperiplant Rebalance

*
Figure 5

A

10 day treatment with

6β-OH-testosterone [nmol mg protein⁻¹ min⁻¹]

solvent
Hyperplant
Rebalance

B

10 day treatment with

2β-OH-testosterone [nmol mg protein⁻¹ min⁻¹]

solvent
Hyperplant
Rebalance

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